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Transition From *In Situ* to Invasive Breast Carcinoma

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TESE DE DOUTORAMENTO APRESENTADA
AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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PATOLOGIA E GENÉTICA MOLECULAR

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TRANSITION FROM *IN SITU* TO INVASIVE BREAST CARCINOMA

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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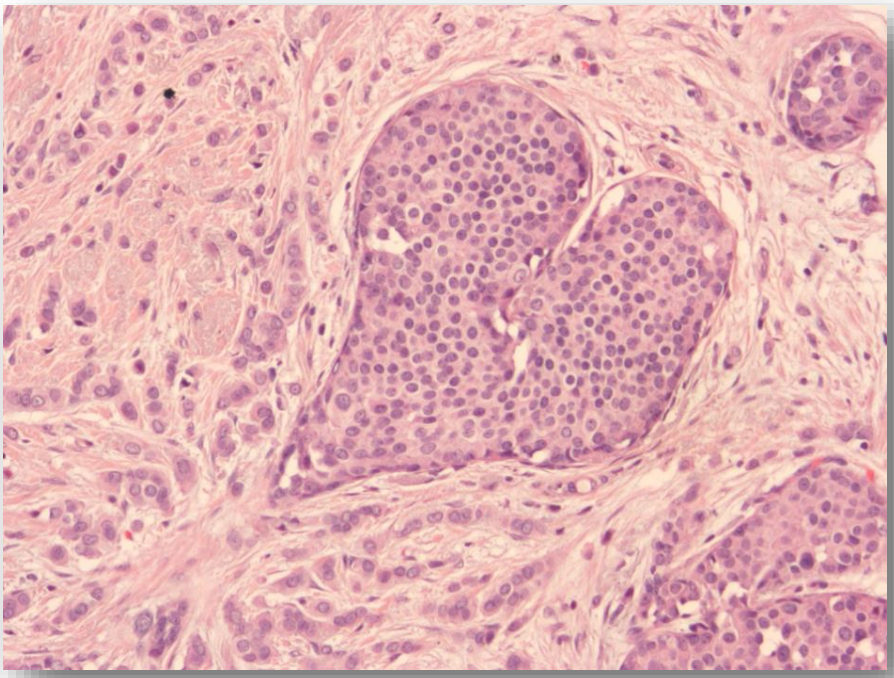

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I. Martins D, Sousa B, Lopes N, Gomes M, Veronese L, Albergaria A, Paredes J, Schmitt F. Molecular phenotypes of matched in situ and invasive components of breast carcinomas. Hum Pathol 2011 Oct;42(10):1438-46.

II. Martins D, Beça FF, Sousa B, Baltazar F, Paredes J, Schmitt F. Loss of caveolin-1 and gain of MCT4 expression in the tumor stroma: key events in the progression from an *in situ* to an invasive breast carcinoma. Cell Cycle 2013 Aug 15: 12(16): 2684-90.

III. Martins D, Paredes J, Schmitt F. Immunohistochemical analysis of progression specific genes in matched *in situ* and invasive ductal carcinomas of the breast. A validation study. (In Preparation).

O artigo IV não faz parte integrante desta dissertação mas foi produzido como um artigo de revisão, que inclui partes da introdução desta Tese:

IV. Martins D, Beça FF, Schmitt F. Metastatic Cancer: Mechanisms and Opportunities for treatment. (Submitted for publication).

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ABBREVIATIONS LIST

ADM	Adrenomedullin
BRCA1	Breast Cancer 1 gene
BRCA2	Breast Cancer 2 Gene
CAF	Cancer Associated Fibroblast
Cav-1	Caveolin-1
cDNA	Complementary DNA
CISH	Chromogenic *In Situ* Hybridization
CK	Cytokeratin
CXCL12	CXC motif chemokine 12
CXCL14	CXC motif chemokine 14
DAB	Diaminobenzidine
DCIS	Ductal Carcinoma *In Situ*
DNA	Desoxyribonucleic Acid
E-cad	Epithelial Cadherin
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor 1
ER	Oestrogen Receptor
FISH	Fluorescence in situ hybridization
H&E	Haematoxilin and Eosin
HER2	Human Epidermal Growth Factor Receptor 2
HER2 OE	Human Epidermal Growth Factor Receptor 2- overexpressing
HRP	Horseradish Peroxidase
IDC	Invasive Ductal Carcinoma
IDC- NOS	Invasive Ductal Carcinoma not otherwise specified
IHC	Immunohistochemistry
LSAB	Labeled streptavidin-biotin-peroxidase complex

MCT4	Monocarboxylate transporter 4;
MMP-11	Matrix Metalloprotease 11
mRNA	Messenger Ribonucleic Acid
PBS	Phosphatase-buffered saline
PgR	Progesterone Receptor
RNA	Ribonucleic acid
P-Cad	Human Placental Cadherin
PgR	Progesterone Receptor
SYT V	Synaptogmin V
TDLU	Terminal ductal-lobular unit
TMA	Tissue Microarray
UBE2C	Ubiquitin- conjugating enzyme 2
WHO	World Health Organization

ABSTRACT

Breast cancer remains a leading cause of death amongst women worldwide. Despite the major diagnostic and therapeutic innovations, the effect on mortality has been modest. One of the factors contributing to this effect is the relative lack of understanding about the natural history of the disease, mainly the progression from *in situ* to invasive breast carcinoma, which is a life-threatening condition. Ductal carcinoma in situ (DCIS) is the precursor lesion for invasive ductal carcinoma (IDC), but the step-wise transformation events that drive its progression are unknown. Although not all the DCIS progress to IDC, up to 50% of cases progress, constituting a challenge in breast tumorigenesis, not only in order to predict which *in situ* lesions will become invasive and the time frame in which that will happen, but also to better tailor the treatment, avoiding the overtreatment or under-treatment associated with DCIS management.

Aiming a deeper understanding into the molecular profiles of *in situ* and invasive breast carcinomas, our first aim was to compare the molecular phenotypes of *in situ* and invasive components of breast cancer coexisting in the same sample. We built a tumor series of 189 cases of matched *in situ* and invasive carcinoma components using tissue microarrays and classified them according to their immunoprofiles, into Luminal A and B, HER2 over-expressing and Basal-like subtypes. The overall concordance on the molecular phenotypes both components was 94%, suggesting that the *in situ* and invasive carcinomas developing in a unique breast cancer patient belong to the same molecular subtype, supporting the *theory of the parallel disease* regarding breast tumorigenesis. This theory defends that a specific subtype of DCIS matches a specific subtype of invasive breast cancer, contradicting the view of a *theory of linear progression*, supporting that tumor progression follows a linear pattern, where low grade DCIS progresses to high-grade DCIS and then to invasive ductal breast carcinoma.

In an attempt to explore the transcriptional program that drives invasion, our second aim was to identify biomarkers that could trigger the progression from *in situ* to invasive breast carcinoma, based on a set of genes that has been previously identified at the mRNA level. In order to find out if some of these genes, such as MMP11, Adrenomedullin, Synaptotagmin V and UBE2C were differentially expressed between DCIS and IDC, we have studied their protein expression by immunohistochemistry in the same series of matched DCIS/IDC. In our series, the levels of protein expression were similar between the *in situ* and invasive components, ranging from 75% to 88% of concordance between both counterparts, suggesting that alterations in these genes can occur prior to invasion.

Actually, during several decades, researchers have only focused their attention in tumor cells and the alterations found in these cells have been the center of breast tumorigenesis. However, the majority of the studies failed to demonstrate significant differences between the expression proteins in the neoplastic cells of DCIS and IDC, suggesting that the alterations in the tumor microenvironment would have a more important role in the progression from an *in situ* to an invasive phenotype than the biology of the tumor cells *per se*. Nowadays, it is widely accepted that close interactions between cancer cells and stroma are known to regulate breast cancer pathways and thus, the determination of differential tumor-stromal metabolic interactions could be an important step in invasiveness. Addressing the role of the microenvironment, our third aim was to investigate in the series of breast cancer samples, including matched *in situ* and invasive components, if there was a relationship between stromal Caveolin-1, a known metabolic protein involved in the progression from DCIS to IDC and MCT4, also involved in metabolism as a transporter of L-lactate from glycolytic cells. MCT4 is associated with Caveolin-1 in triple negative breast cancers, predicting poor clinical outcome. Loss of stromal Caveolin-1 expression in the progression to IDC was found in 75% of the cases. In contrast, MCT4 stromal expression was acquired in 87% of the IDCs. Additionally, when matched *in situ* and invasive carcinomas were compared, a concomitant loss of Cav-1 and gain of MCT4 was observed in the stroma of 75% of the cases, suggesting that alterations in Cav-1 and MCT4 may thus mark a critical point in the progression from *in situ* to invasive breast cancer.

In summary, the results generated in this work indicate that immunhohistochemical profiles do not differ significantly in the transition from *in situ* to invasive breast cancer. Furthermore, the set of genes - MMP11, Synaptotagmin V, Adrenomedullin and UBE2C- previously found to characterize the transition from *in situ* to invasive breast carcinoma, at the mRNA level did not differ also significantly between both components at the protein level. Finally, we found that the microenvironment seems to play an important role in breast cancer progression, since the loss of stromal Cav-1 and the concomitant gain of stromal MCT4 showed significant differences in the transition from *in situ* to invasive carcinoma of the breast.

RESUMO

O carcinoma da mama constitui a principal causa de morte por cancro entre as mulheres em todo o mundo. Apesar dos avanços relativos ao diagnóstico precoce e terapêutica, o efeito na mortalidade é ainda pequeno. Uma das causas deve-se ao conhecimento limitado da história natural da doença, nomeadamente a progressão do carcinoma *in situ* para o carcinoma invasivo da mama. O carcinoma *in situ* (DCIS) constitui a lesão percursora do carcinoma invasivo, contudo a série de eventos que conduz à progressão permanece pouco conhecida. Apesar de nem todos os carcinomas *in situ* progredirem para invasivos, até 50% dos casos pode progredir, constituindo um desafio na carcinogénese mamária, predizer não só quais são as lesões que progridem, mas também a altura em que essa progressão ocorre de forma a adequar o tratamento a cada paciente.

Na tentativa de compreender os perfis moleculares dos carcinomas *in situ* e invasivos da mama, o nosso primeiro objectivo foi comparar os fenótipos moleculares de ambos os componentes, presentes na mesma amostra. Assim, construímos uma série de 189 casos usando "Tissue Microarrays" e classificamos os casos de acordo com os perfis moleculares em Luminal A e B, com sobre-expressão de HER-2 e basal. A concordância global dos fenótipos moleculares entre os componentes *in situ* e invasivo foi de 94%, sugerindo que carcinomas *in situ* e invasivos que se desenvolvem na mesma paciente, têm o mesmo subtipo molecular, suportando a teoria paralela de progressão no carcinoma da mama. Esta teoria sugere que um subtipo de DCIS corresponde a um subtipo de carcinoma invasivo, contrariando a teoria de progressão linear, que sugere que a progressão tumoral ocorre de forma linear, onde o DCIS de baixo grau progride para DCIS de alto grau e posteriormente para carcinoma invasivo.

Numa tentativa de explorar o programa transcripcional que conduz à invasão, o nosso segundo objetivo consistiu em identificar biomarcadores que poderiam desencadear a progressão de carcinoma *in situ* para invasivo, com base num conjunto de genes previamente identificados por mRNA. De forma a perceber se alguns destes genes, como a MMP11, Adrenomedulina, Sinaptotagmina V e a UBE2C estavam diferencialmente expressos no DCIS e IDC, estudamos a sua expressão proteica por imunohistoquímica na mesma série de DCIS/IDC. Os resultados mostraram que os níveis de expressão da proteína foram semelhantes entre os componentes *in situ* e invasivo, variando de 75% a 88% de concordância, sugerindo que essas alterações moleculares podem ocorrer em estádios pré-invasivos.

Durante várias décadas, os investigadores focaram a sua atenção nas células tumorais e as alterações genéticas encontradas nestas células. Contudo, a maioria dos

estudos falhou em demonstrar diferenças significativas na expressão de marcadores nas células do carcinoma *in situ* e invasivo, sugerindo que as alterações no microambiente tumoral poderiam ter um papel mais importante na progressão de carcinoma *in situ* para um fenótipo invasivo do que a biologia das células tumorais. Atualmente é comumente aceite que as interações entre as células tumorais e o estroma regulam as vias de sinalização no carcinoma da mama e portanto a determinação das interações metabólicas tumor-estroma podem constituir uma etapa importante na invasão. Relativamente ao papel do microambiente, o nosso terceiro objetivo consistiu em verificar, numa série de casos de carcinoma da mama, com áreas correspondentes de *in situ* e invasivo, se havia uma associação no estroma entre a Caveolina-1, metabolicamente envolvida na progressão do DCIS para IDC e o MCT4, também associado ao metabolismo devido ao seu papel como transportador de lactato das células glicolíticas. O MCT4 está associado com a Cav-1 nos tumores de mama triplo-negativos, correlacionando-se com pior prognóstico. A perda de expressão da Cav-1 na progressão para o carcinoma invasivo ocorreu em 75% dos casos. Pelo contrário, houve ganho de expressão do MCT4 em 87% no carcinoma invasivo. Adicionalmente, quando comparamos os casos *in situ* com o correspondente carcinoma invasivo, a perda da Cav-1 e o ganho concomitante de MCT4 foi observado em 75% dos casos, sugerindo que as alterações na Cav-1 e no MCT4 possam constituir um ponto crítico na progressão do carcinoma *in situ* para o carcinoma invasivo.

Em conclusão, os nossos resultados sugerem que os perfis immunohistoquímicos não diferem significativamente na transição do carcinoma *in situ* para o carcinoma invasivo. Para além disso, o painel de genes- MMP11, Adrenomedulina, Sinaptotagmina V e UBE2C descritos previamente como diferencialmente expressos na progressão *in situ* e invasivo, a nível de mRNA não diferiam significativamente entre as duas componentes em termos proteicos. Por fim, sugerimos que o microambiente tem um papel importante na progressão da carcinogénese mamária, dado que a perda da Cav-1 e o concomitante ganho de MCT4 no estroma constituem alterações significativas na progressão do carcinoma *in situ* para o carcinoma invasivo da mama.

THESIS OUTLINE

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In Chaper I, a general introduction presents the most current knowledge on breast cancer. It summarizes the epidemiology, risk factors, prognostic factors, therapeutic strategies and classification, devoting particular attention to breast tumorigenesis, mainly to the theories of progression from *in situ* to invasive carcinoma and the parameters that can modulate this progression. A brief description of the importance of tumor microenvironment is also described.

In Chapter II, the rationale and the aims of the thesis are defined whereas, in Chapter III, a description of the material and methods used to perform the studies are described.

Chapters IV, V and VI encloses the three main manuscripts describing the original data presented in this thesis, which were already accepted or in preparation for publication in international peer reviewed journals. Each chapter contain an introduction, the results and the discussion for each proposed aim.

In Chapter VII, an integrated view of the results is presented, with the general discussion.

CHAPTER I

GENERAL INTRODUCTION

1. BREAST CANCER

1.1) EPIDEMIOLOGY

Cancer remains the major public health problem worldwide, where one in three women and one in two men will develop cancer during lifetime, in developed countries [1]. In 2008, there was an estimated 3.2 million cases of diagnosed cancer, with approximately 1.7 million cancer deaths, in Europe [2].

Breast Cancer is by far the most commonly diagnosed cancer and the leading cause of cancer death in women in both developing and developed countries of the world, with an estimated 1.4 million new breast cancer cases and 458.000 deaths in 2008 [3] [2]. Actually, there is an estimative of about 1 million of new cases per year in the world, being the areas of higher risk the ones including populations from North America, Europe and Australia. Conversely, the risk for developing breast cancer is lower in African and Asiatic continents (Figure 1A).

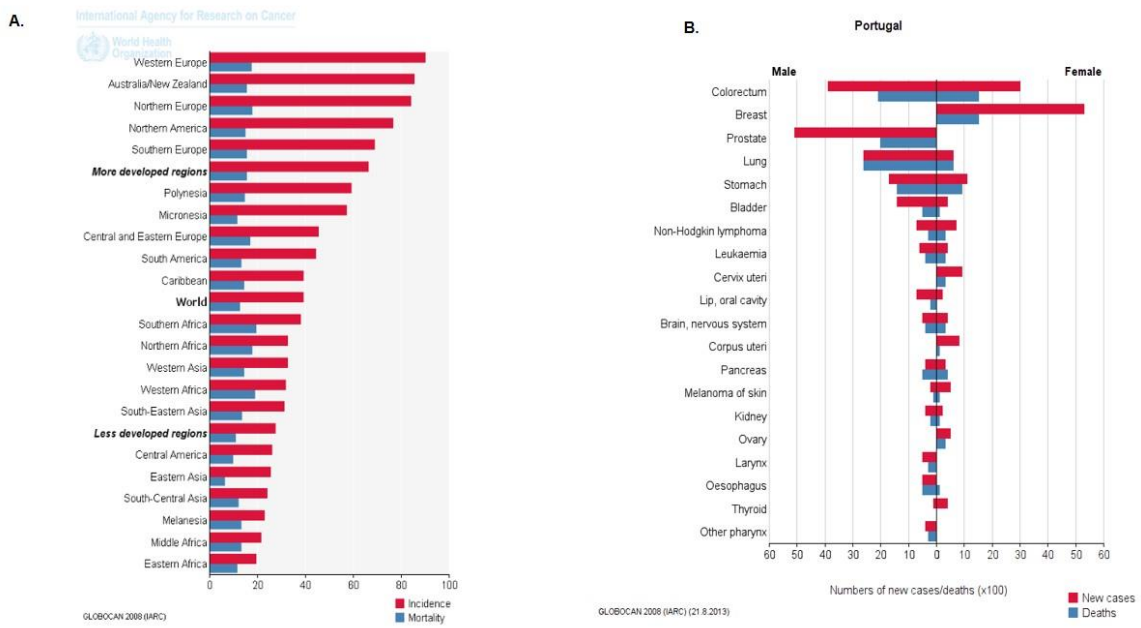


Figure 1: A. Breast cancer estimated age-standardised incidence and mortality rates in the World per 100 000. B. Incidence and mortality rates of the most common types of cancer for both sexes in Portugal - estimates presented for the year 2008. [Adapted from Globocan 2008; Ferlay J et al, 2007]

In Portugal, according with the data from Portuguese League Against Cancer (www.ligacontracancro.pt) [4] breast cancer is also the first leading cause of cancer related deaths amongst Portuguese women, although at a lower rate when compared with the mean established for European Union countries. There are around 5333 new cases every year, with an incidence rate of 49.9 cases per 100.000 people. Mortality rates are in order of 14.4 cases per 100.000 people, corresponding to 4.2 deaths daily (1537 deaths/year), with the estimate age-standardized mortality rate being lower than the European ratio [2] (Figure 1B). This reality has turned breast cancer one of the major interests in the national cancer research field However, since the ninety´s, breast cancer incidence and mortality have steadily decreased, with this decreased representing the progress in early diagnosis, better and more efficient treatment modalities, and a greater awareness and investment in education for early disease detection.

1.2) RISK FACTORS

Breast Cancer has a multifactorial etiology, resulting in a variety of genetic changes and consequently diverse biological behaviors among different patients [5]. The interaction between the environment and the individual genetic profile dictate the susceptibility for breast cancer and several risk factors are associated with breast cancer development. Besides being female, increasing age is the most important risk factor since breast cancer incidence rates double about every ten years [6]. There are also marked differences in the incidence of breast cancer in different places, being the incidence of breast cancer most common among Caucasians living in the colder climates and the more highly industrialized countries of the western hemisphere [7]. The observed differences in breast cancer incidence rates among countries may reflect demographic variations in modifiable risk factors [8]**.** In fact, earlier age at menarche, post-menopausal obesity, physical inactivity, dietary fat and alcohol consumption contributes to increased breast cancer risk [9-11]. Certain breast alterations and findings such as high breast tissue density, high bone mineral density and biopsy-confirmed hyperplasia, especially atypical hyperplasia, represent significant risk factors and increase the probability of malignant development [5, 12]. On the other hand, childbearing and breastfeeding seems to have a reduced risk, with higher protection for early first birth and a larger number of births [11]. Reproductive factors that increase risk include a long menstrual history (menstrual periods that start early and/or end later in life), never having children and having one's first child after age 30.

Among the hormonal influence, a major role has been attributed to the exposure to elevated levels of oestrogens, as has been indicated for a variety of female cancers, namely vaginal and endometrial carcinomas, since oestrogens have effects on cell proliferation and DNA damage, as well as in the promotion of cancer growth [7]. Similarly, it has been shown that the risk for breast cancer development is also increased in women that present high blood and tissue levels of oestrogen and progesterone [8]. Regarding the influence of endogenous hormones, several studies have explored the effect of endogenous serum concentrations of hormones and breast cancer risk. Postmenopausal women with elevated levels of serum estradiol, showed an increased risk for breast cancer development [13]. In fact, post-menopausal obesity, the late menopause (after the age of 55 years) and the early menarche (before than 12 years) are important risk factors, due to the breast exposure to elevated levels of oestrogen. In respect to exogenous hormones, studies concerning the use of oral contraceptives and hormonal therapy for the menopause are still inconclusive, being the last associated with increased risk of breast cancer, especially when comparing its use during short and long periods of time [13] [14].

Environmental and lifestyle factors rather than inherited genetic factors account for most cases of breast cancer; however, the existence of family inherited germline mutations in breast cancer susceptibility genes, like BRCA1 and BRCA2, is a well-established risk factor for breast cancer. In fact, approximately 5% to 10% of all breast cancer cases result from the presence of mutations on these inherited susceptibility genes. Women with a family history of breast cancer, especially in a first-degree relative, have an increased risk of developing the disease themselves [15]. Also in men, approximately 15 to 20% of patients with breast cancer have a family history. BRCA2 mutations predispose men to breast cancer and may account for 4 to 14% of all cases [16].

1.3) PROGNOSTIC FACTORS AND THERAPEUTIC STRATEGIES IN BREAST CANCER

The outcome for women with breast cancer varies widely. Whereas some women have a normal life expectancy, others have only a 10% change of being alive in 5 years after diagnosis. Patient prognosis is mainly determined by pathological characteristics of the primary tumor and the status of axillary lymph nodes.

The pathological examination includes cytological and histological assessment of some classical anatomo-pathologic parameters, which give prognostic and predictive information, essential for the treatment and follow-up of breast cancer patients, such as histological grade and type, axillary lymph nodes status and tumor size [6]. In the absence of

distant metastasis, the lymph nodes *status* represents the major threat to breast cancer patient outcome. Patients without nodal involvement have a 10-year disease free-survival rate close to 70-80%, falling to 35-40% with one to three positive nodes and 10-15% when more than 10 nodes are positive [8]. Tumor size is another powerful independent prognostic marker, with larger size associated with worse outcome. The prognostic and predictive importance of tumor size is of greatest relevance in node-negative breast cancer patients [8]. Women with node-negative carcinomas, with tumors less than 1 cm in size, have a 10-year survival rate of 90%, reducing for 77% when the tumor size is higher than 2 cm [17]. The histological grade provides important independent prognostic data. Several grading systems have been used over the years, and generally include several combinations of tissue architectural arrangement, nuclear features and mitotic rate. Many studies have demonstrated a significant association between overall survival and histological grade, which is now recognised as a powerful prognostic factor and included as a component of the minimum data set for histological reporting of breast cancer [8].

The therapeutic strategies for breast cancer include mainly surgery, chemotherapy and radiotherapy. The primary goal of breast cancer surgery is to remove the neoplastic lesion, as well as the regional axillary lymph nodes, in order to assess the extent of disease spreading and, therefore supporting the decision of therapy regimens. Surgical treatment involves breast-conserving surgery or mastectomy. When conserving surgery is appropriately used for localized or regional cancers, long-term survival is the same as with mastectomy [18]. However, some patients require mastectomy because of large or multiple tumors, or because of a reluctance or inability to undergo radiation therapy after breast-conserving surgery [1]. Systemic adjuvant therapies, like chemo and radiotherapy, given to patients after surgery, is designed to eradicate clinically undetectable microscopic deposits of cancer cells that may have spread from the primary tumor, which usually result in decreased recurrences and improved patient survival [6]. Besides these therapeutic strategies, isolated or in combination with the referred prognostic factors, there are some factors, predictive of outcome, that potentially direct therapies against molecular targets, like nuclear hormone receptors, growth factors and their tyrosine-kinase receptors [19]. The two main molecular biomarkers important for therapeutic management in breast cancer are the hormonal receptors (ER and PgR) and the Human Epidermal growth factor receptor type 2 (HER2). The assessment of these proteins is extremely important, as ER and HER2 are considered prognostic and predictive markers, not only because they allow the stratification of patients for treatment by identifying cases with different outcomes, but also because they select patients that are likely to respond to therapy.

HORMONAL RECEPTORS

ER is a ligand-activated transcription factor that belongs to the superfamily of nuclear receptors for steroid hormones. Current assays use immunohistochemistry to detect hormonal receptors, a finding that is commonly correlated with a low breast cancer histological grade, better patient outcome and is an important predictor of response to hormonal therapy [20] [21]. There are two types of ER: ERα and ERβ. These two receptor subtypes vary in structure, and their encoding genes are on different chromosomes [22]. The tissue distributions of the isoforms also differ, although there is some overlap. Besides ERβ is expressed in breast cancer cells, its involvement in carcinogenesis is controversial, and some studies suggest that in ER-positive carcinomas, the mean ratio ERα/ERβ is higher than in normal tissue [23] [23] [24].

Oestrogen can influence breast carcinogenesis acting as an initiator, causing DNA damage by hydroxylated oestrogen metabolites, or as a promoter, inducing growth of transformed cells [25] [26]. Upon cellular diffusion, oestrogen binds to ER, which alters receptor conformation and activates dimerization. The dimers interact with several coactivators or corepressors in order to modulate transcription of target genes [22].

Since most breast carcinomas are, at least initially, hormone responsive, systemic endocrine therapy is an established strategy for adjuvant breast cancer treatment [27]. Current endocrine therapies of breast cancer are based on three main known mechanisms of action, all of them targeting the ER signalling pathway [20]: antagonizing ER function by competitive binding (selective estrogen receptor modulators), downregulating ER (achieved by pure antiestrogens) and reducing levels of synthesized estrogen (performed by aromatase inhibitors).

Selective oestrogen receptor modulators (SERMs) are molecules that act like agonists of oestrogen in some tissues, but are antagonists to oestrogen action in others [28]. Tamoxifen, the first prototypic SERM [29] was originally developed as an oral contraceptive [30], being recognized as an antioestrogenic and the first-line endocrine agent for breast cancer treatment. Tamoxifen can reduce the risk of breast cancer in women at high risk for developing the disease and is beneficial in pre and post-menopausal women whose tumors are ER positive [31] [32]. Therefore, although tamoxifen causes tumor regression in some women with metastatic disease [33], this range of activity may account for some of the undesirable effects of tamoxifen, such as increased endometrial proliferation and increased risk of endometrial carcinoma [34]. In breast, tamoxifen binds to ER and induces dimerization, but it impairs the binding of the dimers to DNA and inhibits the binding of coactivator proteins. Unfortunately, the treatment with tamoxifen is not effective for more

than 5 years, since development of resistance is a very common event [35]. New SERMs have been developed, like Raloxifene, which has lower toxicity, decreases breast cancer incidence and has no oestrogen-like activity on the uterus [36].

Aromatase inhibitors, which prevent the peripheral tissue conversion of adrenal androgens into oestrogens, resulting in lower oestrogen levels in the circulation and in tumor tissues, have been shown to be superior to tamoxifen and are now incorporated into first line therapy of advanced disease [6] [37]. Most importantly, aromatase inhibitors are effective even in postmenopausal women with low oestrogen concentrations [37] and are also effective in ER-positive breast cancer patients [38], especially when these tumors also express high levels of the oncogene HER2, which is known to increase tamoxifen resistance [39]. Additionally, a recent study demonstrated an increased in overall survival in patients who had switched from Tamoxifen to aromatase inhibitors therapy [35], and these compounds also showed superiority over tamoxifen in the neoadjuvant setting, even challenging chemotherapy with regard to response in a selected group of patients [40] [41].

Pure antiestrogens, such as ICI 182,780, are agents that competitively inhibit the binding of oestrogens to ER, prevent dimerization, promote ER degradation and thereby abolish the transcription of target genes [42]. ICI 182,70, commercially known as Fulvestrant, has been shown to be efficient in the treatment of metastatic ER-positive breast cancer [43], and an appropriate clinical option in ER-positive and HER2 overexpressing tumors [44]. Moreover, ICI 182,780 exceeds other ER targeted therapies, since no side effects have been observed in premenopausal women with metastatic breast cancer, previously exposed to Tamoxifen and aromatase inhibitors [45].

HER2

HER2 is a member of the HER family (or ErbB) of receptor tyrosine kinases, which is involved in proliferation and survival [46]. This family encompasses four growth receptors with a high degree of homology: HER1 to HER4, being HER1 usually designated as EGFR (Epidermal Growth Factor Receptor) [47]. Although HER2 has partial homology to EGFR, a ligand for HER2 has not been identified to date. However, HER2 is transactivated by EGF-like ligands [48], resulting in the formation of EGFR/HER2 heterodimers and, in an analogous way, neuregulins can induce the formation of HER2/HER3 and HER2/HER4 heterodimers. This heterodimerization between HER2 and the other receptors of the family allows the participation of HER2 in signal transduction. HER2 is overexpressed in 25% to 30% of human breast cancers [7] and predicts poor prognosis in patients with primary disease [49]. Gene amplification is the main cause leading to this overexpression in

mammary tumors, although activating mutations have also been described in other cancer models [50]. In order to control the malignant growth effects induced by HER2 overexpression, there has been an attempt to develop drugs that could effectively block the activity of this transmembrane protein.

The targeting of HER2 occurs by the inhibition of the extracellular domain, using monoclonal antibodies and/or by the inhibition of the tyrosine kinase domains, through tyrosine kinase inhibitors [51]. Trastuzumab is a humanised murine monoclonal antibody that was the first genomic research-based product approved for cancer therapy [52]. It displays a potent growth inhibitory effect and significantly improves disease-free and overall survival rates in patients with HER2 overexpressing breast carcinomas [53] [54]. However, the wide range of function mechanisms of trastuzumab give rise to various mechanisms of resistance due to the cross-talk of HER2 with other extracellular domains of HER proteins, resulting in incomplete inhibition and activation of other proliferative pathways [55]. This led to the development of small molecules that bind to the intracellular kinase domain of HER2, thereby inhibiting its activity. Lapatinib is one of these molecules, which inhibits both EGFR and HER2. Some studies have shown that the use of Lapatinib, in combination with other agents, represented survival advantages in patients with HER2overexpressing metastatic breast cancer [56].

1.4) MOLECULAR PORTRAITS

Human breast carcinomas represent a heterogeneous group of tumors diverse in their natural history and their responsiveness to treatment. In fact, patients with identical tumor type and stage of disease can present different responses to therapy and different overall outcomes [57] [58]. The limitations of the current system are based on its inability to take into account the biological prognosis determinants [57]. Based on the above, in the past decade, high throughput microarray technology and gene expression profiling have been applied in breast cancer, in an attempt to further clarify its heterogeneity, allowing the linkage of molecular expression profiles to clinical patient´s outcomes and responses to therapy, generating a tool to better tailor treatment strategies to specific subgroups of patients. Another important implication is that molecular profiling may lead to the identification of new targets for therapy [6] [59] [60].

Microarray-based gene expression profiling led to a working model for breast cancer molecular taxonomy, where clusters of genes, with coherent expression patterns, could be related to specific features of biological variation among tumor samples. Recent cDNA and tissue microarrays studies have showed that breast tumors can be classified into specific

molecular subtypes, distinguished by differences in their gene expression patterns, providing a distinctive portrait of each tumor and the basis for an improved breast cancer taxonomy [49] [58] [61]. Variations in growth rate, in the activity of specific signalling pathways, and in the cellular composition of the tumors were all reflected in the variation of the expression of a specific subset of genes and in the prognosis of the patients [59] [61] [58]**.** In summary, the breast cancer molecular classification distinguishes three main molecular subtypes of breast cancers: the ER positive/luminal-like subtype, a gene expression cluster characteristic of the luminal cells; the HER2 overexpressing subtype, usually associated with the gene amplification of the HER2 proto-oncogene; and the triple negative carcinomas, that comprise mainly “normal breast-like”, claudin-low carcinomas and basal-like, (Figure 2) [62, 63].

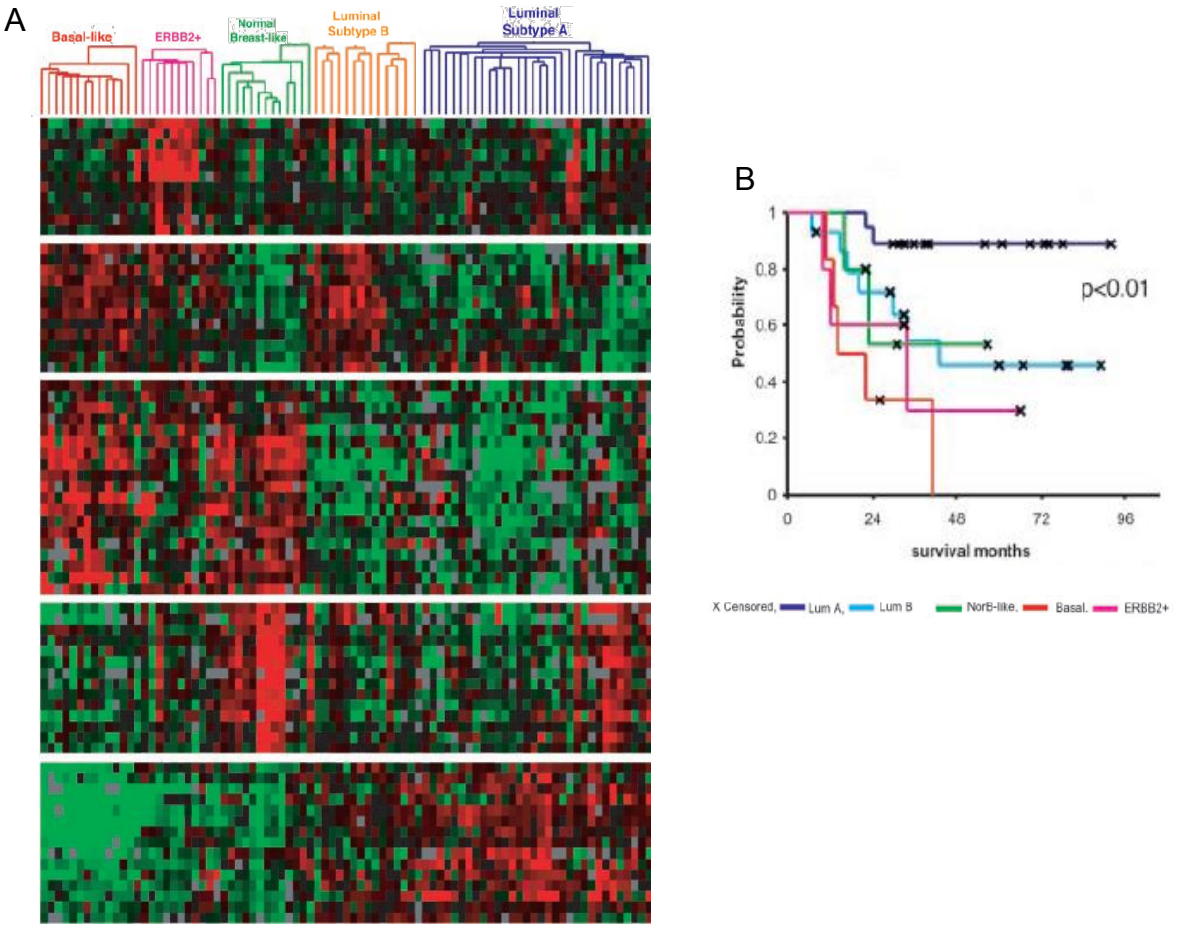


Figure 2: A. Hierarchical clustering of breast tissue samples into five subgroups. Dark blue - luminal A subtype; yellow - luminal B subtype; pink - HER2 overexpressing subtype; red – basal-like subtype and green – normal breast like subtype. B. Overall survival analysis for the five expression-based tumor subtypes based on the classification presented in Figure 2A. Adapted from Sorlie T et al, 2001 [49].

The ER positive/luminal-like molecular subtype, which represents the majority of breast carcinomas (60-75%), is characterized by relatively high expression of genes that are usually associated to ER expression [59] and are generally considered good prognosis carcinomas. Luminal A and Luminal B subtypes can be distinguished by some features, namely HER2 expression and proliferation rates: Luminal B tumors are HER2 positive and display higher levels of proliferation that can be measured by Ki67 staining. Additionally, these tumors express lower levels of ER-associated genes and are associated with worse prognosis than Luminal A tumors [64] [65] [62] [66]. Therapeutic strategies for these subtypes of breast carcinomas involve direct targeting of ER, either by the use of Tamoxifen or the administration of aromatase inhibitors. These tumors are generally well or moderately differentiated, slow growing and well-responsive to endocrine treatments.

HER2 overexpressing subtype comprises ER negative carcinomas that overexpress HER2/neu protein and represent about 20% of all mammary tumors [67]. In the majority of HER2 positive cases, overexpression is due to amplification of a DNA segment including the gene ERBB2 in the 17q21 amplicon [68]. These cancers are usually poorly differentiated, with high proliferation rates and display poor prognosis [49] [62]. However, these can be targeted with the monoclonal antibodies against HER2 (Transtuzumab) or using tyrosine kinase inhibitors, such as Lapatinib.

Triple-negative carcinomas represent 10-17% of all breast carcinomas. They are a heterogeneous group of tumors that lack ER, PgR and HER2 expression, being usually insensitive to endocrine and HER2 therapies. This group of tumors encompasses normal-like, claudin-low and basal-like carcinomas. The normal-like subgroup displays high expression levels of genes associated with adipose tissue, as well as basal cell genes, whereas presents low expression of luminal cell-associated genes. Although these tumors usually cluster together with benign mammary lesions and are usually associated with a better prognosis [62] [69], this group of tumors is quite controversial, since it is considered by several studies as an artefact derived from analysis of tumor specimens with a high proportion of normal tissue contamination [64] [70] [71]. Claudin-low tumors are a recently described molecular subgroup within the triple negative tumors, defined by the downregulation of a cluster of genes involved in cell-cell adhesion, namely Claudins 3, 4, and 7, Occludin and E-cadherin. Furthermore, these tumors exhibit low expression of luminal genes, inconsistent expression of basal-like genes, and high expression of lymphocyte, mesenchymal and endothelial cell markers [72] [73]. Basal-like breast tumors represent one of the most intriguing subtypes, comprising a small proportion of breast carcinomas (about 15%), which exhibit a basal/myoepithelial phenotype, defined by immunohistochemical positivity for basal/ myoepithelial markers [74] [75]. These tumors, that fail to express ER, PgR and HER2, express basal keratins, p63, P-cadherin, EGFR, laminin, α6β4-integrin and

vimentin [76] [77] [78]. This cluster also encompasses the expression of several genes involved in cell cycle, invasion, metastasis and angiogenesis [57], which might explain the association of these tumors with a poor prognosis signature, development of recurrence within the first 5 years after diagnosis, with high proliferative and high grade tumors, short patient survival and high mortality rates [17, 79]. Interestingly, these tumors present a specific pattern of distant metastasis, with increased frequencies in the lungs and brain [80] [81]. When these basal-like breast carcinoma immunoprofiles were compared to familial and sporadic origin, it was observed that basal tumors were mostly associated with familial cases [76]. Actually, it has been described that tumors from BRCA1-mutated carriers share an immunohistochemical profile very similar to that from sporadic basal-type carcinomas (high grade, ER-negative, PgR-negative and HER2-negative). Due to their triple-negative phenotype, currently chemotherapy and radiotherapy for systemic and local control remains the mainstay to treat basal-like cancer, although a high proportion of patients die in a shorter time frame due to metastatic disease.

Recently, the advances in understanding the genomic diversity of breast cancer led to the characterization of a new genome-driven integrated classification of breast cancer, refining the existing classification systems used. The novel classification integrates molecular information on the genomic and transcriptomic landscapes of breast cancer, defining 10 integrative clusters [82]. The integrative clusters were each associated with distinct copy number variations and gene expression changes, clearly demonstrating the heterogeneity present within tumors classified according to ER, PgR and HER2 expression [83]. Furthermore, the 10 groups were associated with distinct features and clinical outcomes [82, 83].

2. BREAST TUMORIGENESIS

2.1) THE MAMMARY GLAND

The mammary gland in humans and other mammals is a dynamic organ that undergoes significant developmental changes during embryonic development, puberty, pregnancy, lactation and involution. The adult female mammary gland consists of a branching tree-like network of ducts, lined by a double layer of epithelial and myoepithelial cells, surrounded by fibroblasts embedded in an extracellular matrix or stroma, mainly composed by a dense fibrous connective tissue, admixed with adipose tissue, and harbouring vascularity (Figure 3). Stroma, besides the structural support provided to the mammary gland, it seems to play an important role in the dynamic induction of the breast gland structure morphogenesis and differentiation [84] [85].

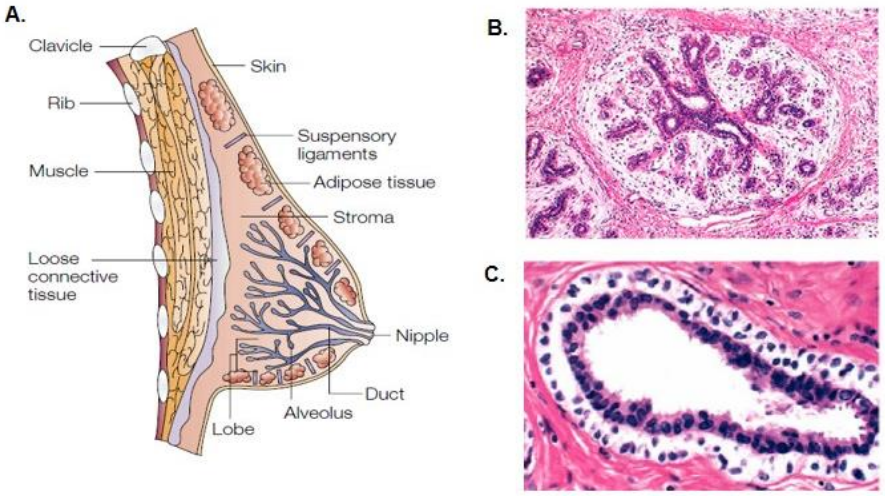


Figure 3: A. Schematic representation of a mature female breast showing the main anatomical structures. Adapted from Ali S et al. 2002; **B.** Histological picture of a normal mature TLDU, composed of acini, surrounded by intralobular connective tissue; Haematoxylin-eosin staining; x100 **C.** Segmental breast duct. The epithelial and myoepithelial cells can be easily identified; Haematoxylin-eosin staining; x1000.

In the ductal-lobular branching system, the lobules of the human breast are organized into 15-20 lobes, which are drained by collecting ducts that converge at the nipple in a radial arrangement [86, 87]. Each lobule in turn is made up of acini (also called alveoli), originating the functional secretory units of the mammary gland, the terminal duct lobular units (TDLUs). These secretory units presents essentially two layers: a sheath of contractile cells containing myofilaments (myoepithelial or basal cells), which are in contact with the

basement membrane, and a second layer of epithelial cells that contours the lumen of the ducts (luminal cells) [88]. Myoepithelial cells are responsible not only for assisting milk ejection, but also for maintaining the normal structure and function of the lobule and basement membrane. Myoepithelial cells are characterized by expressing P-cadherin, α-smooth muscle actin (α-SMA) and basal epithelial cytokeratins (CK5 and CK14). The luminal cells are secretory cells whose function is to produce milk and can be distinguished by the expression of nuclear receptors for steroid hormones oestrogen and progesterone, as well as a subset of epithelial cytokeratins, such as CK8, CK18 and CK19 [89].
Frequently, is at the level of the TDLU, which not only constitutes the functional structure of the breast for milk production, but also is highly responsive to hormonal stimulus occurred during development and maturation processes like pregnancy and lactation [84], that many of the known epithelial benign and malignant lesions are observed [90].

2.2) *IN SITU* BREAST CARCINOMA

In situ breast carcinomas represent a group of malignant lesions that can be confined within the ducts (ductal carcinoma in situ or DCIS) or lobules (lobular carcinoma in situ or LCIS) of the breast.

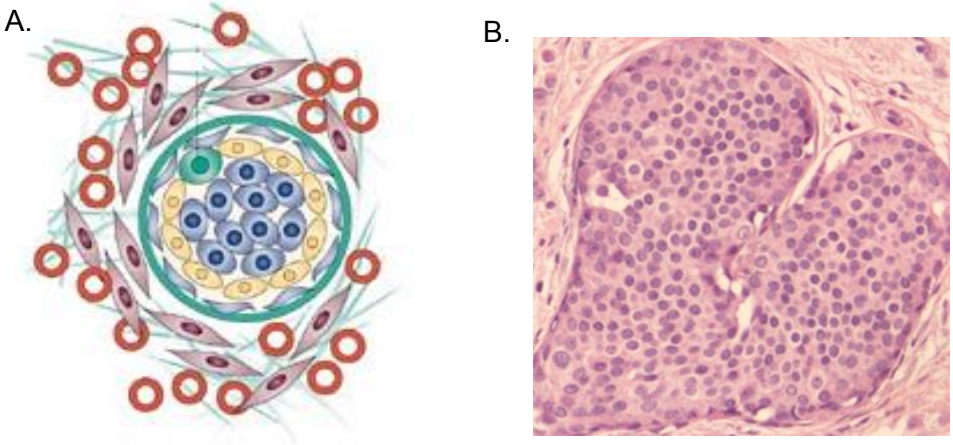


Figure 4: **A.** Schematic *representation of a ductal carcinoma in situ of the breast, Adapted from Kalluri R. Nature Reviews Cancer* **6**, 392-401 (2006); **B.** Haematoxylinn-eosin staining of ductal carcinoma in situ (DCIS), (200x).

This classification was based on the resemblance of the involved spaces to normal ducts or lobules. However, it is now recognized that varied patterns of growths *in situ* are not

related to the site or cell of origin, but rather reflects differences in tumor cell biology, such as whether the tumor cells express the cell adhesion protein E-cadherin or not. Currently, “lobular” refers to carcinomas of a specific type, and “ductal” is more generally used for adenocarcinomas that have no other designation [17]. Besides the nomenclature, both lesions share considerable epithelia proliferation rates associated with malignant cellular features, such as neoplastic proliferation, but without invasion of the physical barrier formed by the basement membrane of the duct or lobule.

Although the evaluation of tumor size and margin involvement appears valuable for the current care of patients, the histologic subtypes of DCIS seem to have influence on its behaviour. Until the last few years, the histological classification for DCIS had been based on architectural features (papillary, micropapillary, cribriform solid and comedo) [91]. However, this division is an oversimplification and does not always stratify patients in those with a high risk of local recurrence versus those with low risk. Nuclear grade is a better biologic predictor than architecture, and therefore it has emerged as a key histopathologic factor for identifying aggressive behaviour [92] [93] [94]. A new DCIS classification was introduced [95], based on the presence or absence of high grade and comedo-type necrosis. High grade nuclear lesions were more likely to recur at a higher rate and in a shorter time period after breast conservation than patients with lower grade lesions [94] [95]. Although there is not an established method for DCIS classification, the parameters used rely on whether the lesion is high nuclear grade or non-high grade, and just then in the presence or absence of necrosis [96].

DCIS is the most frequently breast *in situ* carcinoma and represents a heterogeneous disease with increased incidence after the introduction of breast screening programmes [97]. Before mammography was a routine screening tool, DCIS was rare, representing only 2-5% of symptomatic breast cancers, compared with almost 20% of newly diagnosed symptomatic cases [98]. The widespread use of mammography changed not only the way DCIS was detected, but also changed the nature of the disease. Although the treatment is to prevent recurrence or invasive carcinoma, not all the patients with DCIS will progress to invasive disease, ranging from 25% to 50% depending on the grade of the lesion [99]. Studies concerning the frequency of DCIS in routine autopsy suggests that some non-invasive lesions detected mammographically, and subsequently removed, would not have been of clinical importance, which raises the uncertainties in biological behaviour and in clinical management [100]. Although the risk of death, after any treatment for DCIS is less than 2% after 10 years, recurrence rates vary for different treatments [101] [102].

Concerning the treatment options, mastectomy is an extreme but highly effective treatment for DCIS, if the goal is simply to prevent local recurrence. Most mastectomy series reveal local recurrence rates of approximately 1% with mortality rates close to zero [103]. It

clearly provides a local recurrence benefit but only a theoretical survival benefit. It is indicated in cases of true multicentricity (multiquadrant disease) and when a unicentric DCIS is too large to excise with clear margins. Patients who test positive for BRCA1 or 2, and who develop DCIS, do not have an absolute contraindication for breast conservation, but many of these patients elect bilateral mastectomies. However, mastectomy is an aggressive form of treatment for patients with DCIS, confering no survival advantage and representing an overtreatment for most DCIS patients. Conversely, breast-conserving surgery alone is associated with higher rates of ipsilateral breast tumor recurrence for most subgroups of women and half of local recurrences are invasive disease [104]. A molecular risk profile, through the identification of molecular and biological markers that provide prognostic and predictive information, will be clinically useful if higher-risk patients can be selected for specific treatments; those at lower risk can avoid adjuvant therapies, such as radiotherapy and tamoxifen. Clinical trials have shown that these therapeutic alternative can reduce the risk of invasive recurrence in the ipsilateral breast, but do not represent a benefit in survival to DCIS breast cancer patients [105] [106] [107].

2.3) INVASIVE BREAST CARCINOMA

Invasive breast carcinoma is the most common cancer in women and represents a heterogeneous group of tumors. The diagnosis of DCIS is, in effect, aimed at the prevention of invasive carcinoma, largely because breast cancer has no lethal potential unless it is invasive. It is pathologically defined as malignant cells that have gained access to the stroma of the breast, no longer being delimited by the natural boundary of the basement membrane, the wall that surrounds the entire ductolobular system of the breast [104]. Tumor cells may also gain access to the lymphatic or vascular system, potentially metastasizing to distant sites.

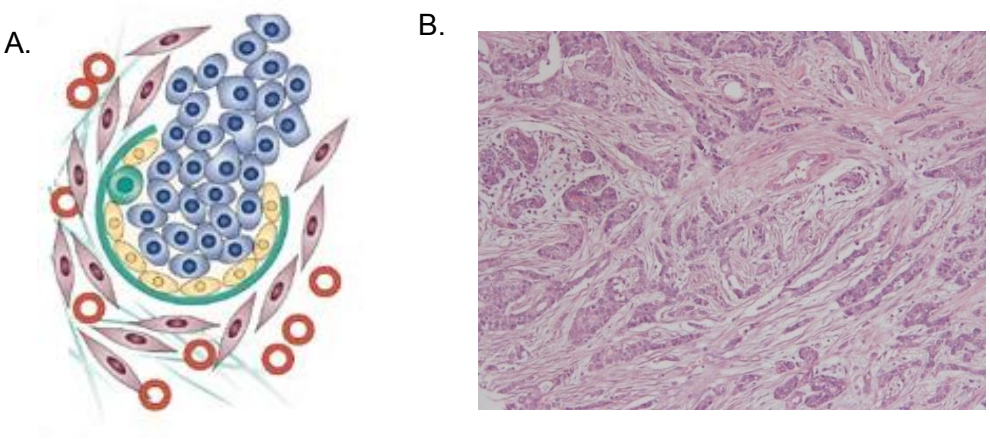


Figure 5: **A.** Schematic representation of an invasive ductal carcinoma of the breast. Adapted from Kalluri R. Nature Reviews Cancer ***6****, 392-401 (2006);* ***B.*** *Haematoxylin-eosin staining of invasive ductal carcinoma of the breast, (100x).*

The invasive ductal carcinoma (ductal carcinoma NOS-not otherwise specified) represents the most frequent histological type, comprising between 70%-80% of all invasive breast cancers. It is a heterogeneous group of tumors that do not exhibit distinct morphological characteristics to be classified in a more specific way, as invasive lobular carcinomas, tubular carcinomas and mucinous carcinomas. The invasive lobular carcinoma, is the second most common type of breast cancer and represents 5 to 15% of all invasive breast cancers and is associated with lobular carcinoma in situ in 90% of the cases. The other types of breast carcinomas, presenting specific morphological characteristics in at least in 90% of the tumor mass, are considered special histological types, which include in breast: tubular, mucinous, cribriform and micropapilar carcinomas, metaplastic carcinoma and medullary carcinoma, among others [8].

Histologic grading is an essential aspect in assessing invasive breast carcinoma and has been repeatedly shown to predict overall and disease-free survival in patients [108]. Well-differentiated invasive ductal carcinomas confer a better prognosis than those that are moderately differentiated, which in turn, confer a better prognosis than those classified as poorly differentiated. The grading schemes commonly used are based on the findings of Bloom and Richardson [109] and include the assessment of three parameters: amount of tubular or glandular formation, degree of nuclear atypia or pleomorfism and mitotic rate. This semi-quantitative method of histological graduation, modified by Elston and Ellis [110], involves the evaluation of three morphological features-the percentage of tubule formation (only the structures exhibiting clear luminal are counted), the degree of nuclear pleomorfism (assessed by reference to the regularity of nuclear size and shape of normal epithelial cells in adjacent breast tissue) and an accurate mitotic count using a defined field area. A numeric scoring system is used and the overall grade is derived from a summation of the scores for the three variables. The score produced by the values is defined by grade as follows: Grade I, well-differentiated (3 to 5 points); Grade II, moderately differentiated (6 to 7 points) and Grade III, poorly differentiated (8 to 9 points).

3. PROGRESSION FROM *IN SITU* TO INVASIVE BREAST CARCINOMA

3.1) THEORIES OF PROGRESSION FROM *IN SITU* TO INVASIVE BREAST CARCINOMA

Predicting which *in situ* lesions will become invasive, as well as the time frame in which that will happen, is one of the most important issues in breast cancer field. Currently, there is a growing interest in developing knowledge regarding the progression from *in situ* to invasive breast carcinoma.

DCIS is thought to be a precursor lesion of ductal invasive carcinoma based on molecular, epidemiological and pathological studies [111]. Although this model is supported by clinical and molecular research [112] [113] [114] [115], only serves as a starting point to understand breast tumorigenesis, since the relation between preinvasive lesions and invasive carcinomas remains unclear [116]. Based on the above, two recent models have been proposed to explain the transition from DCIS to invasive breast carcinoma (IBC).

The *theory of linear progression* [117] [118] [119], suggests that low grade DCIS progresses to high-grade DCIS and just then to invasive ductal breast carcinoma. This model supports that tumor progression follows a linear pattern. However, the behavior of DCIS is inconsistent: only up to 50% of DCIS lesions progress to IBC, and some DCIS have more genetic alterations than some invasive carcinomas [120], which do not fit in this multistep model. Thus, the second model, known as the *theory of parallel disease*, suggests that low-grade DCIS tends to progress to low-grade IBC, whereas high-grade DCIS tends to progress to high-grade IBC. This models implies "commitment" of a subtype of DCIS to a specific subtype of invasive breast carcinoma. Actually, chromosomal-alteration studies support this model, suggesting that, in the progression of DCIS to invasive disease, the histological grade of DCIS corresponds to the grade of subsequent IBC [112] [121]**.** Also, a CGH study demonstrated that 65% of grade 1 tumors had lost the long arm of chromosome 16 compared with only 16% of grade 3 tumors, suggesting that grade 1 tumors do not progress to grade 3 but rather, low-grade and high grade DCIS progress independently [122]. In fact, different stages of progression have been identified for low-grade lesions, and recently, high grade DCIS was recognized as a precursor of high-grade breast cancer, suggesting that low grade and high grade lesions, are distinct pathways in breast tumorigenesis [123, 124].

Moreover, the majority of molecular changes that are observed in invasive breast cancer are already evident in DCIS [125, 126] which is consistent with reports that the tumor is comprised of heterogeneous, independent clones that progress independently and coexist

within the same breast [127]. Moreover, a recent study [128] suggests a convergent phenotype evolution in tumor progression, where several combinations of somatic genetic and/or epigenetic aberrations result in the acquisition of the biological properties required for cancer cells to progress. In fact, this hypothesis constitutes an explanation for the similarities found between *in situ* and invasive carcinomas.

3.2) Genetic Alterations in the Progression to Invasive Disease

High-throughput technologies are been applied to premalignant and preinvasive breast lesions, in order to identify molecular alterations, having an important role in the progression to invasive disease.

Recently, a study using microarray data of 36 breast cancer patients with different pathological stages of disease, revealed a hierarchical portrait of breast cancer progression, identifying genes and pathways for each stage, grade and molecular subtype, suggesting that the heterogeneity of the disease across molecular subtypes is higher than the heterogeneity of the disease progression within a subtype [129]. Using laser-capture microdissection in combination with gene expression profiling in patients with premalignant, preinvasive and invasive breast disease, it was possible to observe that tumor grade was associated with specific gene expression patterns [130]. Several studies are trying to evaluate the gene expression profiles of both *in situ* and invasive components, namely in the identification of specific biomarkers that could trigger the transition from *in situ* to invasive carcinoma, but very few studies analyze *in situ* and invasive components of the same patient. Interestingly, a study analyzing nine matched DCIS/IDC was able to identify 18 genes that were expressed differently between these components [131], showing that MMP11 and UBE2C were upregulated in invasive carcinoma and BPGA1 upregulated in *in situ* counterpart.

Using cells lines, gene expression patterns that correspond to normal, preinvasive and invasive breast cancer allowed the identification of 9 genes that differentiated noninvasive from invasive cell lines [132]. In other recent study [120], it was identified a set of 35 genes that were expressed differentially between DCIS and IBC, mainly involved in signal transduction, cell death and metabolism and 43 genes that discriminate between well differentiated and poorly differentiated DCIS, finding again MMP11 and UBE2C, but also Adrenomedullin, and Synaptotagmin V.

3.3) The role of Myoepithelial Cells in the Progression from *In Situ* to Invasive Carcinoma

The major diagnostic criteria that has been used to differentiate *in situ* from invasive carcinomas is the presence or absence of an intact myoepithelial cell layer (myoepithelial cells from a continuous layer of cells that surrounds the luminal epithelial cells and separates them from the basement membrane and the stroma), which is usually confirmed by performing immunohistochemical analyses against myoepithelial cell-specific genes, such as smooth muscle actin (SMA), p63 or CD10 [133]. However, it remains unknown what leads to the disappearance of the myoepithelial cells in invasive tumors and how this contributes to tumor progression [134]. The exposure of myoepithelial cells to low concentrations of carrageenans (sulphated polysaccharides used in commercial food preparation) leads to cell death, but if the myoepithelial cells are destroyed by these compounds or by other environmental agents is not known [135]. Myoepithelial cells have been recognized as "natural tumor suppressors" and function as gatekeepers of tumor progression [136] [137] due to their inhibitory effect on various neoplastic phenotypes, including tumor cell growth, invasion and angiogenesis [138] [139] [89]. This phenotype was identified through the ability of myoepithelial cells to inhibit the growth and invasion of breast cancer cells in coculture *in vitro* assays and inhibit tumor growth *in vivo* xenograft assays [138] [140] [141]. These effects have been attributed to paracrine factors secreted by myoepithelial cells that exert their effects on the tumor epithelial cells and include ECM proteins, protease inhibitors and several growth factors [134]. In addition, to better understand the role of myoepithelial and stromal cells in the transition from DCIS to IDC, the MCF10DCIS xenograft model, which forms DCIS-like lesions that spontaneously progress to IDC, was co-injected with normal myoepithelial cells, which efficiently suppressed the growth of MCFDCIS xenografts and the transition to invasive carcinoma. These results suggest that the loss of myoepithelial cells promotes DCIS to IDC transition. Also, comparing epithelial, myoepithelial, infiltrating leucocytes, fibroblasts and myofibroblasts, the myoepithelial cells from normal breast tissue and DCIS yielded the highest number of consistently differentially expressed genes [142]. Many of the genes specific for normal myoepithelial cells were absent or dramatically downregulated in DCIS myoepithelial cells, suggesting that in fact these cells appear to be less differentiated and likely have lost some of the functions of normal myoepithelial cells. More importantly, tumor associated myoepithelial cells express higher levels of several basement membrane degrading enzymes including metalloproteases, compared to their normal counterparts [142]. Regarding the progression, many of the genes involved in the normal myoepithelial cell differentiation and function were downregulated, including the ones

encoding laminin and oxytocin receptors, whereas genes that promote tumorigenesis were increased, including CXCL12 and CXCL14.

Although in DCIS, the ducts are still enclosed by the altered myoepithelial cells surrounded by the basement membrane, dramatic changes create a favourable tumor microenvironment. Also, DCIS-associated myoepithelial cells demonstrate altered gene expression and DNA methylation profiles including loss of differentiation markers and elevated levels of pro-angiogenic and invasive genes [143]. The concept of invasive carcinoma includes the degradation of the basement membrane, loss of myoepithelial cells and consequent invasion of the epithelial cells into the stroma, which occurs due to autocrine and paracrine signalling that activated cell migration.

3.4) THE ROLE OF STROMA IN THE PROGRESSION FROM *IN SITU* TO INVASIVE BREAST CARCINOMA

Over the past decades, the major focus of breast cancer research has centred on the tumor cell itself, whereas the role of the non-neoplastic cells of the tumor microenvironment has been largely unexplored. It is now widely acknowledged that accumulation of genetic anomalies contributes to the acquisition of an increasingly aggressive, invasive, or therapy-resistant tumor phenotype [144]. However, phenotypic and genotypic abnormalities in cancer epithelial cells cannot fully delineate tumor phenotypes and clinical behaviour [145]. cDNA microarray profiling and hierarchical clustering analyses have been used to classify breast cancer subtypes and predict clinical outcome [59] [57]. However, comprehensive gene expression and genetic profiling-based studies, comparing *in situ*, invasive and metastatic breast carcinomas have failed, so far, to demonstrate significant differences between different stages of breast cancer progression [130] [146] [131] [147], implying that besides the intrinsic malignant properties of tumor epithelial cells, other factors such as microenvironmental changes may regulate progression to invasion and metastasis.

Cancer has been referred as a “wound that does not heal” and this concept has been redefined at the molecular level as the role of the tumor microenvironment in cancer progression is elucidated [148]. A tumor is much more than clusters of transformed cells standing alone and the epithelial tumor cells can only develop in an aberrant microenvironment composed of altered extracellular matrix and several non-transformed cells, such as fibroblasts, immune cells, myoepithelial and epithelial cells that play a role in the initiation and progression of the neoplasms [149] [150] [151]. Epithelial-mesenchymal interactions in tissue differentiation demonstrated that embryonic mesenchyme strongly influences the terminal differentiation of both embryonic and adult epithelia [152]. In cell

culture, normal mammary epithelial cells in laminin-rich three-dimensional matrix, form acini with a central lumen, become responsive to lactogenic hormones and produce milk proteins enhancing the influence of the ECM and other components of the microenvironment in mammary duct morphogenesis [153]. Early studies demonstrated that the normal mammary microenvironment is capable of "reverting" the neoplastic phenotype of breast cancer cells by inducing cellular differentiation [154] [155]. Still, the changes in microenvironment accompany tumor formation and increased fibroblast proliferation and ECM remodelling are often found adjacent to cancer cells [156]. The tumor microenvironment is not just a passive bystander that simply reacts to the transformed cells, but rather interacts with epithelial cells and has been recognized as a major regulator of carcinogenesis [157], playing a key role in defining tumor behaviour and patient outcome [158]. Gene expression changes occur in cancer-associated stroma and are known to be implicated in the prognosis, as well as in cancer progression [130] [146] [159] [160]. Specifically, evidence from gene expression profiling suggests that the stroma co-evolves with the epithelial compartments during progression [161]. Several *in vitro* and *in vivo* studies, using diverse experimental systems, have demonstrated that the growth, survival, polarity and invasive behaviour of breast cancer cells can be modulated by myoepithelial and various stromal cells, and several genes have been implicated in this process [162] [163] [150, 164]. The influence of stromal cells in the epithelial component (Figures 6 and 7) occurs due to the secretion of several ECM proteins, cytokines, growth factors, proteases and protease inhibitors, constituting an extensive network of cross-talks between cancer cells and the host [165].

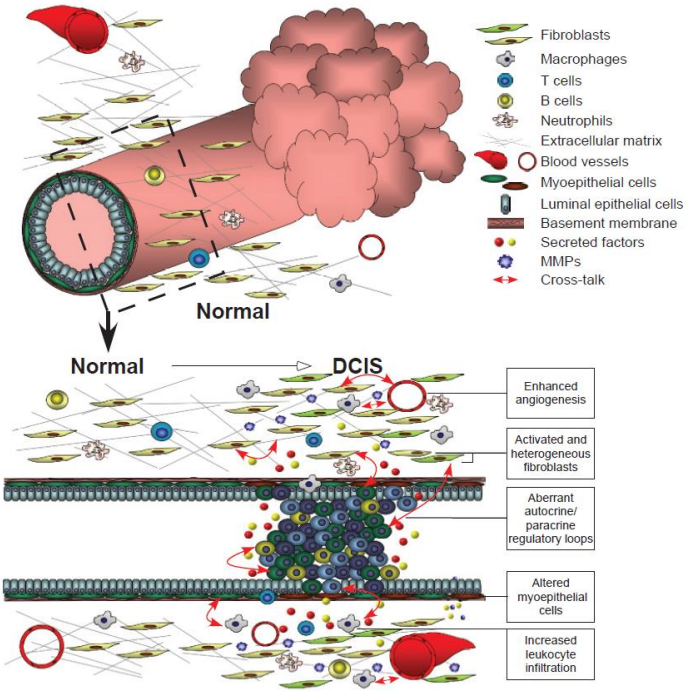


Figure 6: Alterations of the microenvironment from normal duct to *in situ* transition. Considering the alterations of the microenvironment from normal breast tissue to DCIS, the phenotypic and epigenetically altered DCIS myoepithelial cells are surrounded by a still continuous BM. These myoepithelial cells are unable to aid polarization and organize the structure of the normal duct and at the same time, the stroma increases the production of fibroblasts and leucocytes in order to enhance angiogenesis. Growth factors, cytokines, chemokines and MMPs are produced to promote tumor progression. Adapted from Place AE et al, 2011 [166].

Interestingly, when transcriptional profiles of neoplastic cells and stromal cells were compared between DCIS and IBC, more robust changes in gene expression were observed in the stroma [144]**.** Isolating multiple cell types from normal breast, DCIS and IBC lesions and analysing their gene expression profiles, demonstrated that dramatic gene expression changes occur in all cell types, including tumor epithelial, endothelial ad myoepithelial cells, fibroblasts and leucocytes during breast cancer progression [142].

However, some alterations have been identified and relate to processes such as proliferation, ECM remodelling and epithelial to mesenchymal transition [167] [144]. In a related study, using laser capture microdissection and cDNA microarrays to analyse the gene expression profiles of patient-matched samples of normal and tumor-associated epithelium and stroma of DCIS and IDC, although the most dramatic changes in both compartments were observed in the normal to DCIS transition, several ECM-degrading proteases showed elevated expression during *in situ* to invasive carcinoma which may play a role in the destruction of the basement membrane [146] [166].

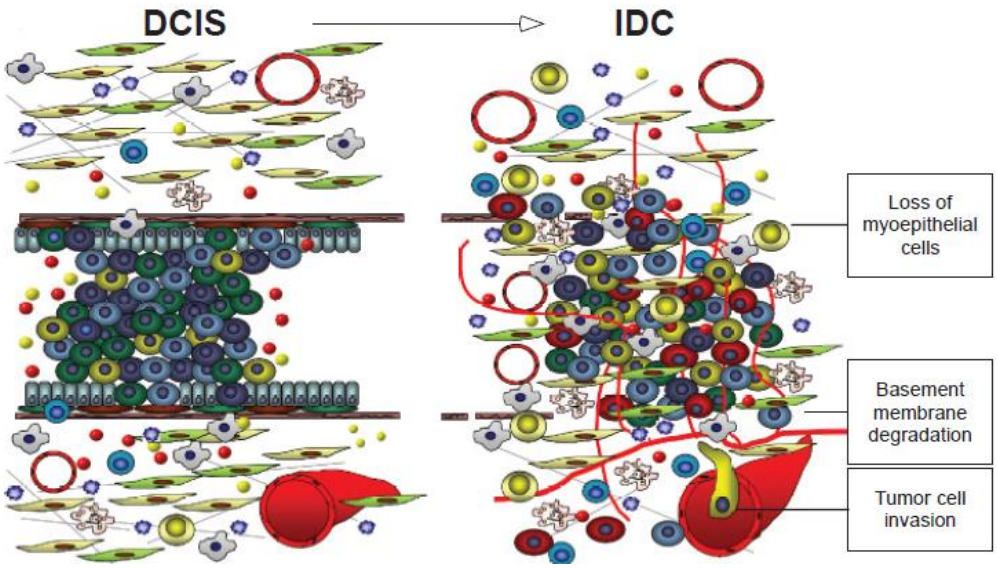


Figure 7: Alterations of the microenvironment in breast cancer progression from *in situ* to invasive carcinoma. Invasive carcinoma occurs through the degradation of the basement membrane, loss of myoepithelial cells and invasion of epithelial cells into the stroma and vasculature, due to the loss of the structural duct and

autocrine/paracrine signaling that activated cell migration. The production of extracellular matrix-degrading proteases by the tumor cells and stromal cells is elevated during the *in situ* to invasive carcinoma transition, leading to destruction of the extracellular matrix such that the tumor cells can invade locally and release more secreted factors. Aberrantly secreted proteolytic enzymes, chemokines, and cytokines continue to attract leukocytes, modulate tumor remodeling. Adapted from Place AE et al, 2011 [166].

Despite the alterations in cell types during tumor progression, other genetic aberrances also happen in breast tumor stroma, including gene copy number changes, loss of heterozygosity (LOH), microsatellite instability (MSI) and point mutations in tumor suppressor genes and oncogenes [168] [169] [170] [171].

The presence of gene expression alterations, but the lack of genetic abnormalities, suggests that epigenetic changes including DNA methylation and chromatin modification are responsible for the abnormal phenotypes of cancer associated stromal cells. Indeed, the analysis of genome-wide methylation profiles identified alterations in DNA methylation patterns not only in tumor epithelial cells but also in stroma fibroblasts and DCIS myoepithelial cells as well, suggesting that the phenotypic changes observed in tumor stromal cells are at least, partially due to epigenetic modifications [143]. Studies in HER2 overexpressing breast tumors [172] and in prostate cancers [173] also demonstrated different methylation patterns in both epithelial and stromal counterparts.

Other key players in stromal component are fibroblasts. Normal fibroblasts are responsible for the maintenance of the extracellular environment through the production and remodelling of the ECM. Cancer-associated fibroblasts (CAFs) differ from the resident fibroblasts of normal tissues in terms of both molecular signatures and functional impact on adjacent epithelial cells [174]. CAFs are themselves heterogeneous with a subset of them sharing markers of contractility with myofibroblasts, such as alpha smooth muscle actin (αSMA), fibroblast activation protein (FAP), desmin, S100A4 and Thy-1 [175] [166], which reflects their acquired motility function. CAF activation is irreversible and in breast cancer, almost 80% of stromal fibroblasts acquire an activated phenotype that manifests by secretion of elevated levels of growth factors, cytokines and metalloproteinases [176]. The origin of CAFs has been actively investigated: one possibility is that they are derived from native interstitial fibroblasts whose phenotype has been modified by aberrant signalling from neighbouring tumor epithelial cells. Alternatively, can differentiate from bone-marrow-derived mesenchymal stem cells that are recruited to the tumor though endocrine stimulation by tumor-derived factors [166]. CAFs are commonly found in the cancer stroma [176] and promote tumor formation [177] and metastasis [178] in human breast cancers. They are essential for ECM deposition and remodelling through the synthesis of several ECM

components and of ECM-degrading proteases, such as matrix metalloproteinases (MMPs). Using gene-expression profiling of laser capture-microdissected tumor stroma from 53 breast cancer patients allowed the identification of a stroma-derived prognostic signature, an independent prognostic factor that strongly predicts clinical outcome [159]. A similar approach was used for predicting chemotherapy resistance in breast cancer, underscoring the important role of the tumor stroma as a determinant of clinical outcome and resistance [160].

The prevailing model of breast tumor progression is tumor epithelial cell-driven, since tumor cells have acquired genetic changes and demonstrate genomic instability, where the cells with the most aggressive phenotype are selected through clonal selection. However, this model has been questioned due to several aspects. Genetic alterations in tumor stroma, although controversial, raises the possibility that clonal selection occurs in nonepithelial cells as well, where the tumor microenvironment may play an active role in driving tumor progression. On the other hand, the identification of global gene expression changes and epigenetic alterations together with the fact that the genetic background of the host influences metastatic behaviour, emphasizes the concept that progression is a “driving force” with several stakeholders. Several models of *in situ* to invasive progression have been proposed (Figure 8), focusing on the “seed” or the “soil” theory [134]. The “escape” model suggests that genetic changes and clonal selection in combination will give rise to a population of tumor epithelial cells with an ability to invade out of the duct and spread into stroma. The “release” model highlights the role of the stroma and suggests that the phenotypic changes of myoepithelial cells, infiltration of leucocytes and fibroblasts will work together and lead to the disruption of the basement membrane [134] [145]. To corroborate this last model, studies demonstrating increased cancer risk in patients with chronic inflammatory disease and decreased risk among users of antiinflammatory drugs, address the role of genetically modified stroma in tumorigenesis [179]. Also, focal breakdown of myoepithelial cell layer and basement membrane at sites of white blood cell infiltration have also been observed in DCIS [180], creating an escape route for the cancer cells [181].

Because changes in the tumor epithelial cells are likely to induce microenvironmental changes, the combination of both models, including the changes in both epithelial and stromal cells could explain progression to invasion. However, clarifying the “chicken or egg” dilemma about what comes first, if the development of a genetically distinct tumor epithelial cell clone or the myoepithelial cell layer disruption will help in understanding the mechanisms that underlying the progression from *in situ* to invasive breast carcinoma.

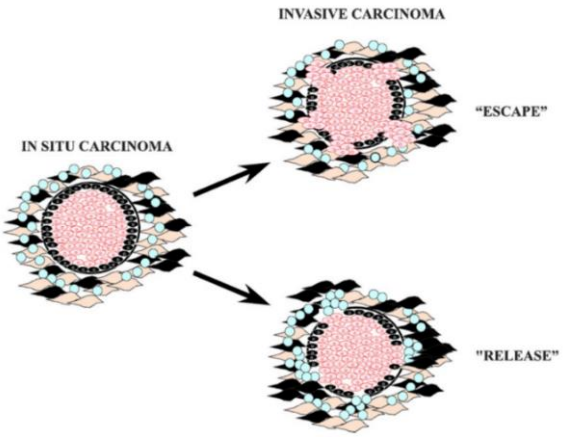


Figure 8: Hypothetical model depicting two views of the progression from *in situ* to invasive breast carcinoma. Adapted from Polyak K et al, 2006 [134].

CHAPTER II

RATIONALE AND AIMS

RATIONALE AND AIMS

Breast cancer remains a leading cause of death amongst women worldwide. One of the factors contributing to this effect is the relative lack of understanding about the natural history of the disease, mainly the progression from *in situ* to invasive breast carcinoma.

The general aim of the work reported in this thesis was to understand the mechanisms of progression from *in situ* to invasive breast carcinoma. Thus, making use of a unique large series of matched *in situ* and invasive breast cancer cases, the studies were performed in order to assess the following three specific aims:

i. **To determine if there are differences between the tumor molecular profiles of matched *in situ* and invasive breast carcinomas**

   It has been demonstrated that the greatest alterations in gene expression are seen among the different histological grades of breast cancer. It has also been suggested that the expression of several tumor markers correlates with grade, but not with the distinction between in situ and invasive breast cancer. Based on the above and after the classification of the different cases into molecular profiles, the purpose is to understand if the transition from *in situ* to invasive breast carcinoma is (in)dependent from the molecular profile.

ii. **To validate by immunohistochemistry, the expression of specific genes that have been previously described as important in the transition from *in situ* to invasive breast cancer**

   It is of great interest to understand the transcriptional program that drives invasive growth and several studies have been trying to identify genes that would mark the transition from *in situ* neoplastic cells to migrating invasive cells. In order to find out if these genes, previously described as discriminating between *in situ* and invasive components, are effectively differentially expressed in the counterparts, we aim to study these proteins, by immunohistochemistry in our series of matched *in situ* and invasive components.

iii. To determine the role of the tumor microenvironment in the step-wise transformation from *in situ* to invasive breast cancer

It has been demonstrated that interactions between the neoplastic cells and the tumor microenvironment may play an important role in breast tumorigenesis. Also, microenvironment participates in tumorigenesis even before tumor cells invade the stroma, and it may play an important role in the transition from pre-invasive to invasive growth. Therefore, we aim to investigate in our series of breast cancer samples including matched *in situ* and invasive components, if there was a relationship between stromal Cav-1 and MCT4, in the progression from *in situ* to invasive carcinoma.

CHAPTER III

MATERIALS AND METHODS

1. TUMOR SAMPLES AND TUMOR BANK CONSTRUCTION

A tumor bank consisting in 189 formalin-fixed paraffin-embedded samples, harbouring in situ and invasive breast carcinomas in the same block were collected from the archives of the Pathology Institute of Araçatuba, São Paulo, Brazil. Patient´s ages ranged from 27 to 96 years old and they were diagnosed from 1996 to 2006. Patient information concerns the age of the patients at the time of diagnosis, the histological grade and the lymph nodes status for in situ and invasive counterparts.

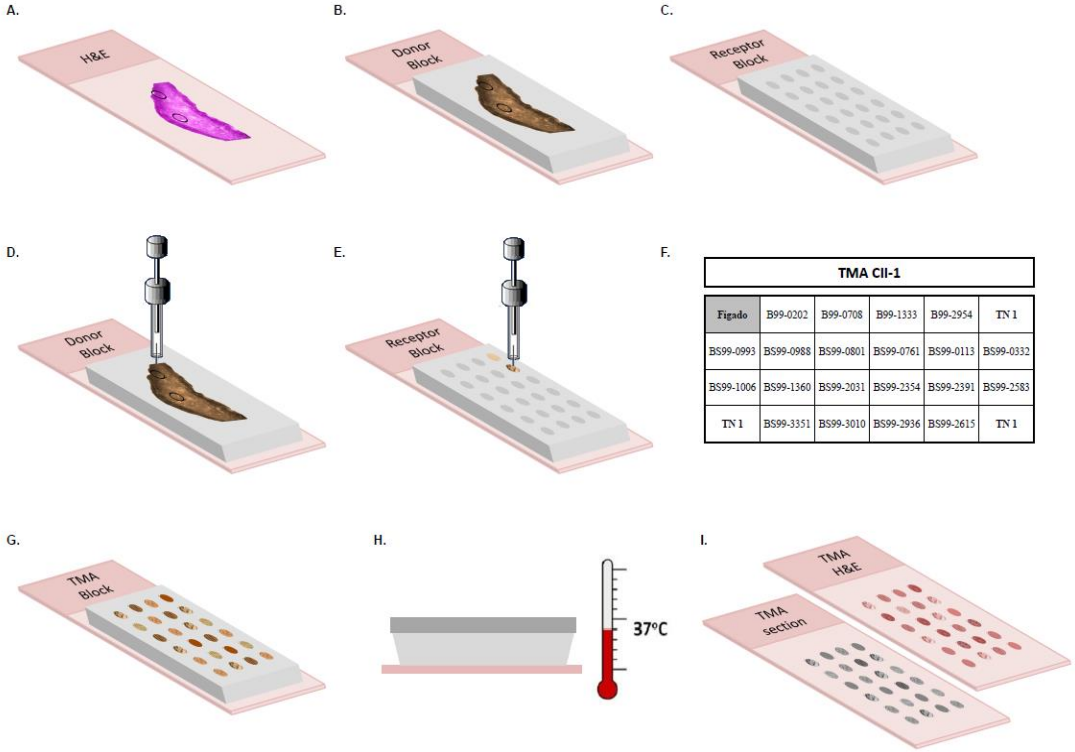
The DCIS samples were subdivided into 3 groups: low, intermediate, and high grade, according to the nuclear grade and the nuclear grade and the extent of necrosis, as previously published [96]. Briefly, tumors harboring nuclear grade 3 were all considered high grade, whereas tumors with nuclear grade 1 or 2 with necrosis were considered intermediate grade, and those of nuclear grades 1 and 2 without necrosis were considered low grade. Invasive breast cancers were classified according to the method described by Elston and Ellis [182], as grade I, II, or III.

All the data was included in an Excel (Microsoft) datasheet, preserving the patient´s anonymity. The study was conducted under the national regulative law for handling of biological specimens from tumor banks, being the samples exclusively available for research purposes in retrospective studies.

2. TISSUE MICROARRAY CONSTRUCTION

The 189 in situ and invasive breast carcinomas were arrayed in 22 tissue microarray (TMA) blocks. For the construction of the TMAs, representative areas of the different lesions were carefully selected on the H&E (haematoxylin and eosin) and marked on individual paraffin blocks. In order to guarantee that the immunostaining results correspond to the appropriate case, a grid was generated, containing the original histological number of the patient. The grid also includes 3 non-neoplastic breast tissues as controls and one core of a non-breast cancer sample (we have used liver), in order to orientate the TMA block. Using a Tissue Microarray workstation (TMA builder, Labvision, USA), two tissue cores (2 mm in diameter) were obtained from each selected specimen- the donor block, and inserted into a recipient paraffin block, each containing 24 cores, arranged in a 4x6 sector.

In order to homogenise the paraffin of the receptor block and to bind the cores into the block, minimizing the loss of the cores during the TMA sectioning, the TMAs were kept at 37ºC during approximately 3 hours. The block was carefully covered with a glass slide adhered to the cores to obtain a homogenized block with a flat surface suitable to be sectioned. The blocks rested at room temperature and the glass slide was removed. Then, 2 to 3-µm tissue sections were performed and adhered to a coated glass slide (Superfrost Plus ®, Gerhard Menzel, Braunschweig, Germany). An H&E-stained section from each block was reviewed to confirm the presence of morphological representative areas of the original sections.



TMA CII-1

Figado	B99-0202	B99-0708	B99-1333	B99-2954	TN 1
BS99-0993	BS99-0988	BS99-0801	BS99-0761	BS99-0113	BS99-0332
BS99-1006	BS99-1360	BS99-2031	BS99-2354	BS99-2391	BS99-2583
TN 1	BS99-3351	BS99-3010	BS99-2936	BS99-2615	TN 1

Figure 9: Stepwise construction of a tissue microarray. **A**. Representative tumor areas were selected on haematoxylin and eosin-stained sections and **B.** marked on paraffin blocks; **C**. The receptor block; **D.** A core of tissue was extracted from the marked area of the donor block; **E.** The extracted tissue is placed in the receptor block; **F.** the TMA grid were the cases are placed; **G**. Placing the tissues in receptor block was repeated to create the organised rows and columns according to the predefined TMA plan; **H.** Once the TMA was complete, the receptor block was slightly melted in order to bind the cores into the block and **I.** sequential tissue sections (2-3µm) were cut and adhered to a coated glass slide.

3. IMMUNOHISTOCHEMISTRY

IMMUNOHISTOCHEMISTRY TECHNIQUE

After the deparaffinization of the tissue sections in a xylene substitute (Clear Rite 3®, Richard- Allan Scientific, Kalamazoo, MI, USA) and hydration though a decreasing series of alcohol concentrations, the epitope retrieval was performed using heat-induced at 98ºC in a water-bath during 30 minutes, using commercially available antigen unmasking solutions: citrate buffer solution 1:100, pH=6,0 (Labvision, Fremont, CA, USA) or ethylenediaminetetraacetic (EDTA) solution 1:10, pH=9,0 (Novocastra, Newcastle, UK), or by proteolytic enzyme digestion using a solution of pepsin A in distilled water (4g/L, Sigma Aldrich, St. Louis, MO, USA) for 30 minutes at 37ªC. After the respective antigen retrieval and washes in phosphate buffer solution (PBS), endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution (Panreac, Spain) in methanol (Sigma-Aldrich, USA) during 10 minutes. The slides were then incubated in a blocking serum (LabVision, USA) for 15 minutes and then incubated with the respective primary antibodies. Information regarding primary antibodies, dilution and suppliers, as well as antigen retrieval and respective detection systems are described in Table 1. After the incubation with the primary antibodies and the washes, the slides were incubated with a strepatividin-biotin-peroxidase complex (Labvision) or with a secondary antibody associated with HRP-labelled (horseradish peroxidase) polymer (Envision®- Dako, Carpinteria, CA, USA), with additional amplification capability. The slides were revealed with diaminobenzidine (DAB) chromogen (Dako) and counterstained with Mayer´s haematoxylin (Richard-Allan Scientific), dehydrated in a crescent concentration of alcohol, clarified with xylene substitute (Clear Rite 3®) and then cover-slipped using a permanent mounting medium (Zymed, USA). Positive and negative controls were also included in each run, in order to guarantee the reliability of the assays.

To molecularly characterize *in situ* and invasive breast carcinomas, we evaluated the expression of ER (clone SP1, Neomarkers, Fremont, CA, USA), PgR (1A6, Novocastra, Newcastle UK), the tyrosine kinase receptors HER2 (SP3 Neomarkers) and EGFR (31G7 Zymed, San Francisco, CA, USA), the basal markers P-cadherin (clone 56, Transduction Labs), CK5 (clone XM26, Neomarkers) and the proliferation marker KI67 (clone SP6, Neomarkers).

To study the proteins that potentially discriminate between the *in situ* and invasive components of breast carcinomas, specific antibodies for MMP-11 (clone SL3.05, Santa

Cruz), Synaptotagmin V -SYTV (polyclonal antibody, Abcam), Adrenomedullin- ADM (clone HTA171/E8, Abcam) and Ubiquitin carrier protein 2 (UBE2C, clone AB-209, BostonBiochem).

In order to study the metabolic alterations in breast tumorigenesis in this series, Caveolin-1 (clone 2297, BD Biosciences) and Monocarboxylate Transporter 4 (MCT4, clone H-90, Santa Cruz) immunohistochemistry was performed.

Table 1: Specific antibodies and conditions used for Immunohistochemistry

Antibody	Clone	Manufacturer	Time of incubation (min)	Dilution	Antigen retrieval	Detection system
ER	SP1	Neomarkers	60	1:100	Citrate Buffer	HRP polymer
PgR	1A6	Novocastra	60	1:40	Citrate Buffer	HRP polymer
HER2	SP3	Neomarkers	30	1:80	Citrate Buffer	LSAB
P-cad	56	Transduction	60	1:50	Tris-EDTA solution	HRP polymer
CK5	XM26	Neomarkers	60	1:50	Tris-EDTA solution	LSAB
EGFR	31G7	Zymed	60	1:100	Pepsin A solution	HRP polymer
Ki67	SP6	Neomarkers	60	1:300	Citrate Buffer	HRP polymer
MMP-11	SL3.05	Santa Cruz	60	1:100	Citrate Buffer	HRP polymer
SYTV	PAB	Abcam	60	1:200	Citrate Buffer	HRP polymer
ADM	HTA171/E8	Abcam	120	1:50	Citrate Buffer	HRP polymer
UBE2C	AB-209	BostonBiochem	60	1:100	Citrate Buffer	LSAB
Cav-1	2297	BD Bioscienses	60	1:50	Citrate Buffer	HRP polymer
MCT4	H-90	Santa Cruz	60	1:500	Citrate Buffer	HRP polymer

IMMUNOHISTOCHEMISTRY EVALUATION

The evaluation of the immunohistochemical results was performed by a pathologist (FS). ER and PgR nuclear expression was evaluated according with 2 parameters: intensity ranked from 1 to 3 (1 – weak, 2 – moderate, 3 – strong), and extension ranked from 1 to 10 (1 – 0-10% cells, 2 – 11-20% cells, 3 – 21-30% cells, 4 – 31-40% cells, 5 – 41-50% cells, 6 – 51-60% cells, 7 – 61-70% cells, 8 – 71-80% cells, 9 – 81-90% cells, 10 – 91-100% cells), using the H-score method [183, 184]. The scores for intensity and extension were multiplied and the cases were considered negative when the score was below 4 and positive from 5 to 30.

HER2 expression was evaluated according to the DakoCytomation Hercept Test® scoring system [185]. Cases with no staining or less than 10% of the cells stained were considered negative and cases with incomplete membranar staining in more than 10% of cells were considered 1+. Cases with more than 10% of cells with weak to moderate complete membrane staining were considered 2+. Cases were considered positive (overexpression) when immunostaining was classified as 3+. If a case is classified as 2+ by IHC, FISH analysis was performed to determine if the tumor had HER2 amplification. If amplification was confirmed, the tumor was classified as positive. If the tumor did not demonstrate amplification, it was considered negative. Cases 1+ were also considered negative. EGFR staining was also classified according to the Hercept Test scoring system. However, breast carcinomas were considered positive whenever the immunostaining was 2+ or 3+. Concerning Ki-67, the quantification of cell proliferation was measured with the public available web application software ImmunoRatio [186]. Tumors with an unequivocal nuclear staining in more than 14% of the cells were classified as high proliferative, whereas tumors with less than 14% of positive cells were considered low proliferative [187]. We considered positive the cases with membranous staining for P-cad and cytoplasmic staining for CK5 in at least 10% of the neoplastic cells [76, 77, 188].

The classification into the different molecular subtypes of breast carcinoma was performed: tumors with positive hormone receptor (ER and PgR), negative for HER2 as well as with low proliferative index were considered Luminal A, whereas the ones with positive hormone receptors and positive for HER2 or with a high proliferative index (Ki67 positive) were considered Luminal B [76, 77, 187, 189, 190]. Breast carcinomas were considered HER2 overexpressing whenever the immunohistochemical reaction was classified as 3+ or when gene amplification was confirmed by Chromogenic *In Situ* Hybridization (CISH) in the 2+ cases, as previously described [191]. Based on the above, cases lacking hormone receptors and with overexpression of HER2 were classified as HER2 overexpressing

tumors. Tumors that were negative for ER, PgR and HER2 and positive for EGFR or CK5 or P-Cad were considered Basal-like. Cases that lacked expression of all tested markers were considered “unclassified.”

To evaluate immunostaining intensity for MMP11, SYT5 and ADM, a numeric score ranging from 0 to 3 was used, reflecting the cytoplasmic intensity as follows: 0, no reactivity; 1, weak reactivity; 2, moderate reactivity; and 3, intense reactivity, as previously described [192, 193]. The score 0 was considered negative, whereas scores 1, 2 and 3 were considered positive. The quantification for UBE2C was performed with the public available web application software ImmunoRatio [186]. This methodology was based on fact that this marker is nuclear such as Ki67 or ER, and this quantification system has already been validated for their expression. The cases without expression of UBE2C were considered negative, whereas the cases that showed expression (more than 1%) were considered positive.

Cav-1 and MCT4 expression in stroma were evaluated using the previously described methodology [194, 195]. In summary, Cav-1 and MCT4 were semi-quantitatively scored as negative (0, no staining), weak (1, either diffuse weak or strong staining in less than 30% of stromal cells per core) or strong (2, defined as strong staining in 30% or more of the stromal cells) [196].

4. Statistical Analysis

For statistical analysis, StatView 5.0 (SAS Institute Inc., Cary, North Carolina, USA) was used in order to perform contingency tables and chi-square tests and also to estimate the associations between the staining pattern of the different antibodies used, as well as between other clinicopathological features. In all statistical analyses, a significant level of 5% was considered.

CHAPTER IV

MOLECULAR PROFILES OF *IN SITU* AND INVASIVE BREAST CARCINOMAS

PAPERS RELATED TO THIS CHAPTER:

Martins D, Sousa B, Lopes N, Gomes M, Veronese L, Albergaria A, Paredes J, Schmitt F. Molecular phenotypes of matched *in situ* and invasive components of breast carcinomas. Hum Pathol. 2011 Oct;42(10):1438-46.

1. INTRODUCTION

Breast cancer is the most common cancer in women, with more than one million cases occurring worldwide annually [197]. Despite significant diagnostic and therapeutic innovations, the effect on the mortality rate has been modest. One of the factors contributing to this limited success is the relative lack of understanding of the the natural history of this disease [198]. For example, the transition from *in situ* to invasive carcinoma is still a poorly understood event [199].

Nowadays, it is widely stated that the natural history of breast cancer involves progression, through clinical and pathologic stages [111, 199], from premalignant hyperplastic breast lesions, with or without atypia, to carcinoma *in situ* and invasive carcinoma [117, 118, 200]. On the basis of molecular, epidemiologic, and pathologic studies, ductal carcinoma *in situ* (DCIS) is thought to be a precursor lesion of ductal invasive carcinoma [111]. Although this model is supported by clinical and molecular research [112-115], it is only a start point to understand breast tumorigenesis, as the relation between preinvasive lesions and invasive carcinoma remains unclear [116]. From the available data, two models have been proposed to explain the transition from DCIS to invasive breast carcinoma (IBC). The first one, the *theory of linear progression* [117-119], suggests that low grade DCIS progresses to high-grade DCIS and then to invasive ductal breast carcinoma. This model implies that tumor progression follows a linear pattern. However, there is evidence that some *in situ* carcinomas never progress for invasion and that some DCIS have more genetic alterations than some invasive carcinomas [120], a finding which does not fit in this multistep model. Consequently, a second model of breast cancer tumorigenesis has been proposed: the *theory of the parallel disease*, wherein low-grade DCIS tends to progress to low-grade invasive ductal breast cancer, whereas high-grade DCIS tend to progress to high-grade invasive breast cancer [116]. In this model, a specific subtype of DCIS matches a specific subtype of invasive breast cancer.

Gene expression profiling is known to be a powerful tool for identifying tumor molecular profiles and for correlating gene expression profiles with outcome in breast cancer [120]. In addition, it has been also an important tool to explore the transcriptional program that leads to invasion, comparing *in situ* and invasive carcinomas. Recently, Dalgin *et al.* [129] studied 36 breast cancer patients with different pathological stages of disease and revealed a hierarchical portrait of breast cancer progression, identifying genes and pathways for each stage, grade and molecular subtype. These authors suggested that the heterogeneity of the disease across molecular subtypes is higher than the heterogeneity of

the disease progression within a subtype, suggesting that tumors with different molecular profiles are in fact distinct diseases.

Several studies have concentrated on the identification of specific biomarkers that could define the subtypes of *in situ* and invasive breast carcinomas [76, 77, 201]. Our group and others demonstrated that is possible to translate the molecular classification, using immunohistochemistry (IHC) and tissue microarrays (TMAs) [77], where estrogen and progesterone receptors (ER and PgR) and Human Epidermal Growth Factor Receptor 2 (HER2) expression identify Luminal A, B and HER2 overexpression subtypes, whereas tumor protein 63 (p63), Cytokeratin 5 (CK5) and P-Cadherin (P-cad) allow the identification of basal-like tumors [76]. Recently, Paredes *et al.* [77] also demonstrated the importance of P-cadherin and CK5 as useful adjunct markers to distinguish basal-like subtype among the *in situ* carcinomas.

However, it was never determined whether the *in situ* and invasive carcinomas that develop in a particular breast cancer patient belong to the same molecular subtype or are different entities belonging to different molecular profiles.

In this study, our aim was to compare the molecular phenotypes of *in situ* and invasive components of breast cancer in the same sample, using IHC, TMAs and a specific panel of biomarkers, previously described by our group [76, 77].

2. RESULTS

We performed IHC on each set of the 22 TMA slides for ER, PgR, HER2, P-cad, CK5, EGFR and Ki-67. Tables 1 and 2 summarize the clustering of a total of 189 immunohistochemically interpretable cases to allow sample characterization into one of the five previously described molecular subtypes. The molecular classification was made in an isolated way for each of the tumor components (*in situ* and invasive) in the same block.

EVALUATION OF THE *IN SITU* COMPONENT

Among the *in situ* component, we observed that 63% of all tumors were considered Luminal A, whereas Luminal B and HER2 overexpressing subtypes comprised 15% and 12% of the cases, respectively. Basal-like tumors represented 7% and the ones with null phenotype/unclassified were 3% (Table 1).

Table 1: Frequencies of the immunohistochemically defined subtypes of *in situ* and invasive breast cancers for the tested markers.

Subtype	Frequency in situ component, n (%)	Frequency invasive component, n (%)
Luminal A	120/189 (63)	116/189 (61)
Luminal B	28/189 (15)	31/189 (16)
HER-2 overexpressing	23/189 (12)	23/189 (12)
Basal-like	13/189 (7)	14/189 (8)
Unclassified	5/189 (3)	5/189 (3)

Because Luminal cancer subtypes (A and B) were defined as positive for hormonal receptors (ER and/or PgR), the percentage of positive cases for these two immunohistochemical markers was extremely high, as expected, with a higher prevalence for ER positivity when compared with PgR positive cases (Table 2). For the Luminal A cancer subtype, 95% and 66% of the cases were ER and PgR positive, respectively; whereas, for Luminal B, 100% were positive for ER and 61% were positive for PgR. As initially defined, all Luminal A tumors were negative for HER-2, and Luminal B were positive for this marker. In the group of negative cases for hormonal receptors, all the cases overexpressing HER2 were included in the HER-2 overexpressing cancer subtype, being the triple negative ones (negative for ER, PgR and HER2) divided into Basal-like or unclassified,

according to the positivity for P-cad, CK5 and EGFR basal markers. Among the Basal-like tumors, P-cad was the most prevalent basal marker, with 92% of the cases being positive, whereas only 23% and 38% of the cases were positive for EGFR and CK5, respectively.

Although basal markers are most commonly expressed in basal-like tumors, these can also be present in other cancer subtypes, but in a lower frequency. Concerning EGFR, although there were almost no positive cases in Luminal A and B subtypes, 10% of HER-2 overexpressing tumors also expressed EGFR. Also CK5 was expressed by 17% of the HER2 overexpressing *in situ* carcinomas, whereas only 3% and 7% of the tumors classified as Luminal A or B, respectively, showed CK5 expression. P-cad expression was also highly found in HER2 overexpressing tumors, being positive in almost half of the cases (48%). Concerning the Luminal cancer subtypes, P-cad expression was more abundant in Luminal B (14%) than in Luminal A (8%).

Concerning cell proliferation indexes, addressed by Ki-67 staining, Basal-like tumors were the ones showing higher values (28%), followed by Luminal B (19%).

Table2: Comparison of molecular subtypes and biomarkers for *in situ* and invasive components

		Luminal A		Luminal B		HER-2 overexpressing		Basal-like		*P*	
		In situ	Invasive	In situ	Invasive	In situ	Invasive	In situ	Invasive	In situ	Invasive
ER	+	114 (95%)	108 (93%)	28 (100%)	31 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	$P \leq .0001$	$P \leq .0001$
	−	6 (5%)	8 (7%)	0 (0%)	0 (0%)	23 (100%)	23 (100%)	13 (100%)	14 (100%)		
PR	+	79 (66%)	79 (68%)	17 (61%)	13(42%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	$P = .0002$	$P = .0001$
	−	41 (34%)	37 (32%)	11 (39%)	18 (58%)	23 (100%)	23 (100%)	13 (100%)	14 (100%)		
HER-2	+	0 (0%)	0 (0%)	15 (54%)	14 (45%)	23 (100%)	23 (100%)	0 (0%)	0 (0%)	$P \leq .0001$	$P \leq .0001$
	−	120 (100%)	116 (100%)	13 (46%)	17 (55%)	0 (0%)	0 (0%)	13 (100%)	14 (100%)		
EGFR	+	1 (1%)	1 (1%)	0 (0%)	0 (0%)	2 (10%)	2 (9%)	3 (23%)	3 (21%)	$P = .004$	$P = .001$
	−	119 (99%)	115 (99%)	28 (100%)	31 (100%)	21 (90%)	21 (91%)	10 (77%)	11 (79%)		
CK5	+	4 (3%)	2 (2%)	2 (7%)	0 (0%)	4 (17%)	5 (22%)	5 (38%)	5 (36%)	$P = .001$	$P \leq .0001$
	−	116 (97%)	114 (98%)	26 (93%)	31 (100%)	19 (83%)	18 (78%)	8 (62%)	9 (64%)		
P-cad	+	9 (7%)	9 (8%)	4 (14%)	4 (13%)	11 (48%)	11 (48%)	12 (92%)	13 (93%)	$P \leq .0001$	$P \leq .0001$
	−	111 (93%)	107 (92%)	24 (86%)	27 (87%)	12 (52%)	12 (52%)	1 (8%)	1 (7%)		

When we studied the association between the *in situ* histological grade and molecular cancer subtypes (Figure 1), we found that Luminal A tumors were frequently classified as low grade (49%), while the majority of Luminal B carcinomas were classified as intermediate grade (53%); HER2 overexpressing and basal-like cases were more often considered of high grade (86% and 77%).

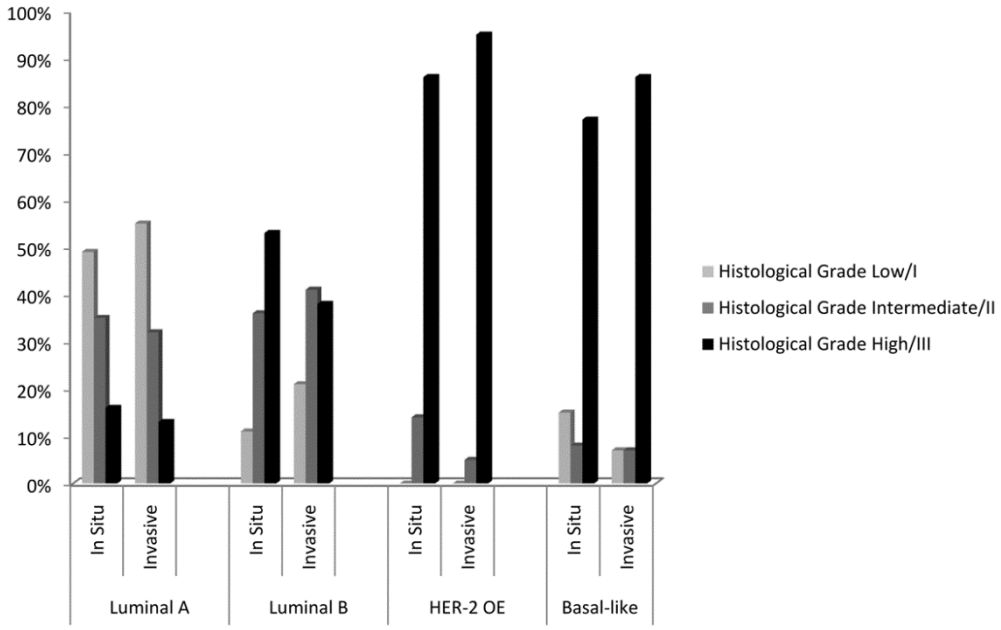


Figure 1: Comparison of histological grade among molecular subtypes (Luminal A, B, HER-2 and Basal) *in situ* and invasive components. All the correlations were statistical significant (p≤0. 05).

EVALUATION OF THE INVASIVE COMPONENT

For the invasive component (Table 1), the Luminal A subtype represented 61% of all the tumors. Luminal B and HER-2 overexpressing invasive tumors corresponded to 16% and 12%, respectively, while Basal-like tumors comprised 8% of the cases. Only 3% of the invasive carcinomas were classified as null phenotype/unclassified.

For the Luminal A cancer subtype, 93% and 68% of the cases were ER and PgR positive, respectively; whereas, for Luminal B, 100% were positive for ER and 42% were positive for PgR. Again, all Luminal A tumors were negative for HER-2 as expected, and 45% of Luminal B cases were positive for this marker. The invasive carcinomas overexpressing HER2 and negative for hormonal receptors were included in the HER2 overexpressing cancer subtype. In triple-negative Basal-like invasive tumors, as described for the *in situ* component, P-cad expression was the most prevailing basal marker, with 93% of positive cases, whereas only 21% and 36% of the cases were positive for EGFR and CK5, respectively (Table 2).

When we studied the expression of basal markers in cancer subtypes other than the Basal-like, we found results similar to the ones described for the *in situ* component of this breast cancer series. Concerning EGFR, exactly the same frequencies were found: 1% and 0% of the cases expressed this receptor in Luminal A and B subtypes, respectively, whereas 9% of HER-2 overexpressing tumors co-expressed these two tyrosine-kinase receptors. CK5

was expressed by 22% of the HER-2 overexpressing invasive carcinomas, whereas only 2% or none of the tumors classified as Luminal A or B, respectively, showed CK5 expression. Again, P-cad expression was highly expressed in HER-2 overexpressing tumors (48%), but only expressed by 8% of Luminal A and 13% of Luminal B invasive breast carcinomas.

For Ki-67, the tumors included in the Basal-like and Luminal B subtypes had the highest proliferative indexes (29% and 25%, respectively). Regarding the histological grade (Figure 1), we found that Luminal A invasive tumors were often considered grade I (55%), whereas Luminal B lesions spread from intermediate (41%) to high grade (38%); once more, HER-2 overexpressing and Basal-like tumors were more regularly classified as grade III (95% and 86%, respectively).

COMBINED EVALUATION OF THE *IN SITU* AND INVASIVE COUNTERPARTS IN THE SAME PATIENT

Most cases (93%) maintained the molecular classification, when the *in situ* and invasive components were compared (Figure 2).

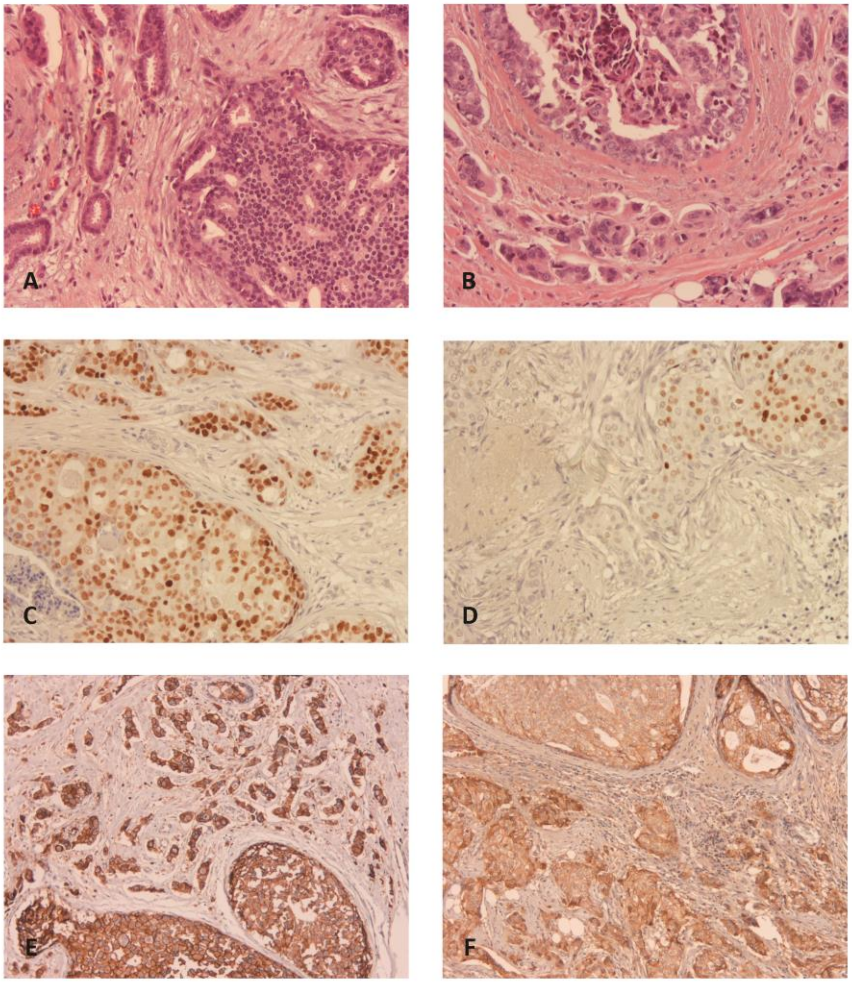


Figure 2: Expression of proteins studied by IHC on TMAs for *in situ* and invasive components. **A** and **B**, HE staining of Low/I and High grade/III histological grade, respectively (x200); **C** Positive ER expression (x200); **D** Lost of ER expression in invasive component (x200); **E** HER-2 staining (x100); **F** P-cad staining (x100).

There were just 13 cases (7%) in which the 2 areas were classified differently (Table 3). One of the cases was unclassified for the *in situ* component (negative for all the markers tested), but Basal-like in the invasive counterpart, due to P-cad expression in this fraction. Independently of the classification, this tumor was graduated as high grade in both components. Other case was classified as an *in situ* Luminal A carcinoma, but was unclassified in the invasive component, due to the lack of expression of both hormonal receptors (ER and PgR) (Figure 2D).

Interestingly, although both components were graduated as intermediate grade, the proliferative index was different between the *in situ* and invasive components in this case. The invasive counterpart had higher proliferative index when compared with the in situ counterpart. Four cases were classified as Luminal B for the *in situ* component, but Luminal A for invasive counterpart due to the low proliferative index in the invasive component, except one case that is due to lack of HER-2 also in the invasive counterpart. Interestingly in this case, the loss of expression was accompanied by alterations in the histological grade, from high grade *in situ* to grade II in the invasive counterpart.

Finally, 7 cases that were classified as *in situ* Luminal A carcinomas were then classified as Luminal B in the invasive counterpart due to higher proliferative index in the invasive component. Besides the increase in cell proliferation, no alterations were noticed in histological grading.

Table 3: Discordant molecular classifications between *in situ* and invasive components

No.	In situ component	Invasive component
7	Luminal A	Luminal B
4	Luminal B	Luminal A
1	Luminal A	Unclassified
1	Unclassified	"Basal-like"

In general terms, we can conclude that there are no important modifications of the breast cancer molecular classification in the majority of the cases, when the transition from an *in situ* to an invasive carcinoma occurs in a breast cancer patient. However, when we compared individually the expression of the different biomarkers tested (Table 2), we could find subtle differences between both components, which can add some biological information to the *in situ*/invasive transition. In the Luminal A cases, besides the increase of proliferative rate in the invasive component in 7 cases, just 3 cases showed P-cad expression in the invasive component. No alterations in hormonal receptors were found between *in situ* and invasive transition. In Luminal B tumors no alterations were found for ER expression, when the *in situ* and invasive components were compared. However, there were 4 cases that lost PgR expression in the invasive carcinoma, with the transition from a high grade *in situ* carcinoma to a grade II invasive tumor in two cases; in the remaining two, there were no alterations in grading. The other differences were in basal markers, such as CK5 and P-cad, with loss of 7% and 3% of expression from *in situ* to invasive tumors, respectively. Regarding the HER2 overexpression cases, 7 did not show the expression of any basal marker, whereas 14 cases showed concomitant expression of EGFR, CK5 or P-cad together with HER2. From these, P-cad was the most prevalent. There were 3 cases

which gained the expression of basal-markers in the transition from the *in situ* to the invasive carcinoma, namely two with CK5 and one with P-cad. Only this last case changed the histological grade when both components were compared (from an *in situ* intermediate grade to a grade III invasive tumor). In Basal-like subtype, the majority of the cases were P-cad positive in both components (eleven cases). However, there was one case which lost P-cad in the invasive fraction but, since it expressed CK5 in the invasive component; its molecular classification did not change.

Molecular breast cancer subtypes of *in situ* and invasive tumors did not vary with the histological grade of these lesions ($p<0,0001$ and $p=0,0002$ for *in situ* and invasive counterparts, respectively). High grade lesions were associated with HER2 overexpressing and Basal-like phenotype, both in the *in situ* and invasive components. Low grade lesions were frequently associated with Luminal A phenotype. In Luminal B phenotype, the *in situ* component was more frequently high grade (53%), while the invasive counterpart was intermediate (41%).

As mentioned above, the only cases with alterations in molecular classification were not accompanied by differences in histological grade. However, there were some alterations in histological grade in some individual cases: nine cases, with histological classification of intermediate lesion in the *in situ* component were grade III in the invasive counterpart, whereas five cases classified as *in situ* low-grade, were grade II lesions when we analyzed the invasive component. In cases where there was a decrease in the histological grade, twelve cases classified as high grade in the *in situ* component were grade II in the invasive counterpart; one case was high grade in the *in situ* component and grade I in the invasive one and ten cases classified as intermediate grade in the *in situ* component were grade I in the invasive area.

3. DISCUSSION

Two main branches in breast tumorigenesis have been distinguished: one supports the multi-step model of breast cancer and the other the theory of parallel disease, where a specific subtype of DCIS matches a specific subtype of invasive breast cancer [116]. In 1997, Grupta *et al.* [202], studying 300 patients with invasive breast carcinoma associated with DCIS, demonstrated that the degree of differentiation of DCIS was correlated with the grade of the invasive carcinoma and the clinical outcome. They also showed that patients with invasive breast cancer in that series also displayed the same genetic mutations as patients with preinvasive and invasive lesions. In fact, recent data [203] demonstrate that the most dramatic alterations in gene expression patterns occur during the transition from normal breast tissue to DCIS [125, 126], and not from *in situ* to invasive transition. In contrast, Tamimi *et al.* [29], studying 272 DCIS and 2249 invasive independent tumors, showed that *in situ* and invasive phenotypes were differently prevalent. These authors found an increased prevalence of Luminal B and HER2 overexpressing profiles among DCIS tumors. However, analyzing independent series of in *situ* and invasive tumors [17, 18], no differences were found in molecular subtype prevalence. So, probably, the higher percentage of HER-2 phenotype in DCIS in Tamimi *et al* [204] series was due to mammographically screened population and does not represent a basis of progression to invasive tumors.

The great advantage of our series of 189 breast carcinomas, which was characterized by several immunohistochemical markers, relies on the existence of *in situ* and invasive components in the same sample. We classified the *in situ* and invasive tumors into four main molecular subtypes (Luminal A, Luminal B, HER2 overexpressing and Basal-like). When both components were compared, we verified that there were no significant differences in molecular classifications of *in situ* and invasive tumors which lead us to conclude that different molecular subtypes have different progression forms, evolving low grade *in situ* tumors to low grade invasive tumors and high-grade *in situ* tumors to high grade invasive tumors [129].

Differences in molecular profiles from the *in situ* to invasive carcinoma were observed in only 13 cases, which seems more likely to be attributable to technical immunohistochemical issues than a reflection of changes in the tumor biology. One case classified as Luminal A in the *in situ* component lost ER expression and became unclassified in the invasive component. Another case, which did not express any of the markers used for classification (unclassified) in the *in situ* component, gained expression of P-cad in the

invasive component and could be characterized as having Basal-like phenotype. In the remaining 11 cases, the changes were from Luminal A to B and vice-versa and these alterations can be attributed to the fact that the criteria for classification of Luminal B subtype are not well established. Although some Luminal B tumors can be identified by their expression of HER2, the major biological distinction between Luminal A and B is the proliferative signature, including genes such as Ki-67. Chang M and collaborators [187], using 14% as a cut-off, supported Ki-67 as a well –established cell proliferation marker in cancer and emphasized its role as a biomarker candidate for identification of Luminal B tumors. We also used this cut-off, which allowed us to distinguish some Luminal B tumors that the standard biomarker panel (ER, PgR and HER2) did not identify. Interestingly, and although the percentages are really close to Luminal B subtype, the Basal-like tumors had higher proliferative index when compared with the other subtypes, in both *in situ* and invasive components (28% and 29% respectively). Among these fractions, the invasive one had a higher proliferative rate, which can be associated with increase of cell proliferation when invasion occurs, and with the poor prognosis associated with this molecular subtype.

An association between histological grade and molecular phenotype has been demonstrated, with low-grade invasive tumors usually having the Luminal A phenotype, whereas high grade tumors are more prevalent among HER2 overexpressing and Basal-like subtypes [204, 205]. Moreover, the HER2 overexpressing and basal-like subtypes are associated with poor prognosis. In our series, in *in situ* breast cancers, HER2 and Basal-like subtypes were more frequently high grade than low grade or intermediate (86% and 77% respectively). These results were consistent for invasive tumors, as 95% of HER2 and 86% of Basal-like present high histological grades. It was interesting to note the percentages of Luminal B histological grade in DCIS and invasive component, where intermediate /II and high grade prevail in both, with 36% and 54% for *in situ* component, as well as 41% and 38% for invasive counterpart, respectively. This similarity between intermediate /grade II and high grade is probably due to the cut-off used that enriched our series in Luminal B cases.

We also looked for cases that showed alterations simultaneously in biomarker expression and in histological grade and found eight cases. It is important to say that these alterations were not accompanied by alterations in the molecular classification. Two cases graduated as intermediate *in situ* component were grade III in the invasive component, accompanied by gain and loss of P-cad, respectively. Other two cases, with the concordant loss of PgR expression classified as high grade *in situ* component, were grade II in the invasive counterpart. One case also lost PgR expression, but changed from intermediate *in situ* to grade I in invasive counterpart. It is also interesting that we had two cases that lost PgR expression simultaneously and one basal marker, P-cad or CK5, and were graduated

as high grade *in situ* and as grade II in the invasive component. Finally, the last case lost CK5 and change from intermediate to grade I.

We have shown that the prevalence of the molecularly defined phenotypes did not differ significantly between DCIS and invasive breast cancers; probably, the molecular alterations which drive invasion occur before the morphological modification of the lesion [147, 206]. Dalgin *et al.* [129] also confirmed that the cancer phenotype develops early (in early hyperplasia or ductal carcinoma *in situ* stage) and each subtype progresses along its own specific pathway, as if each was a distinct disease.

In conclusion, with this work, we showed that is possible to identify different immunohistochemical profiles of *in situ* and invasive breast cancer, using a small panel of biomarkers (ER, PgR, HER2, EGFR, CK5, P-cad and Ki-67) and that the technique of TMA is useful, efficient and reliable to the characterization and sub-classification of a large number of cases. Concerning the comparison of *in situ* and invasive components, we found that in 176 (93%) of the 189 cases, the molecular classification was identical in the 2 components, which supports the theory of the parallel disease; that is, that in the progression of most breast cancer cases, there is a commitment of the *in situ* subtype carcinoma to a specific subtype of invasive carcinoma. Otherwise, the finding supports the view that the molecular phenotype is established at the DCIS stage. Although there has been an improvement in understanding the pathways of breast tumorigenesis, little is known about the mechanisms associated with the transition from *in situ* to invasive carcinomas. More than just genetic alterations in the tumor cells, the codependency between epithelial cells and the stroma can regulate tumor progression. Recently, it was demonstrated that myoepithelial cells can have a particular role in tumor invasion. Studying normal myoepithelial cells and the ones associated with DCIS, Schnitt et al. [206, 207] demonstrated that the last ones differ substantially from the normal, showing down-regulation of genes involved in the normal function of cells and upregulation of genes associated with invasion. There is an immediate need to characterize new molecules that not only uncover the molecular biology of *in situ* carcinomas and its transition to invasive breast cancer, but also the transcriptional program that drives invasive growth of each molecular subtype.

CHAPTER V

IMMUNOHISTOCHEMISTRY VALIDATION OF SPECIFIC PROTEINS DESCRIBED AS IMPORTANT IN THE TRANSITION FROM IN SITU TO INVASIVE BREAST CARCINOMA

PAPERS RELATED TO THIS CHAPTER:

Martins D, Paredes J, Schmitt F. Immunohistochemical analysis of progression specific genes in matched in situ and invasive ductal carcinomas of the breast. A validation study. (In Preparation).

1. INTRODUCTION

Predicting which lesions will become invasive, as well as the time frame in which the progression from *in situ* to invasive carcinoma will occur, is one of the major focus in breast cancer research. Therefore, there is a significant interest in identifying molecular events driving invasive progression, in order to identify new molecular targets that could trigger cancer progression at early stages [144].

Recently, Dalgin et al [129], using microarray data from 36 breast cancer patients with different pathological stages of disease, were able to reveal a hierarchical portrait of breast cancer progression, identifying genes and pathways for each stage, grade and molecular subtype, and concluding that tumor molecular profiles are actually distinct diseases. Ma et al. [130] using laser-capture microdissection and gene expression profiling of premalignant, preinvasive and invasive breast disease, suggested extensive similarities at the transcriptomic level among the distinct stages of progression, as well as concluded that gene expression alterations conferring the potential for invasive growth were already present at the preinvasive stages. Accordingly, using a series of 189 patient-matched *in situ* and invasive tumor samples, we showed 94% of concordance between *in situ* and invasive component molecular profiles, suggesting there is a commitment of the *in situ* carcinoma subtype to a specific subtype of invasive carcinoma in the progression of most breast cancer cases, supporting the view that the molecular phenotype is established at the preinvasive stage [208].

Besides this knowledge, and in an attempt to explore the transcriptional program that leads to invasion, some studies have focused their attention in the identification of specific biomarkers that could trigger the transition from *in situ* to invasive carcinoma, evaluating the gene expression profiles of both ductal carcinomas *in situ* (DCIS) and invasive ductal carcinomas (IDC) [120, 130-132, 147, 199, 209] (although only few compared both components within the same breast tumor). Analyses of chromosomal aberrations by array-based comparative genomic hybridization (aCGH) could not discriminate DCIS and IDC from the same patients [210-212], showing that these are closely related not only on the basis of their gene expression but also on their gene copy number aberrations [128].

However, some studies have described significant genetic alterations between DCIS and its matched invasive counterpart. Hannemann et al. [120] identified a gene expression classifier of 35 genes which were differentially expressed between *in situ* and IDC, like Matrix Metalloproteinase 11 (MMP11), Synaptotagmin V (SYT5), Adrenomedullin (ADM), and Ubiquitin carrier protein 2 (UBE2C). Shuetz et al. [131], analyzing nine matched DCIS/IDC,

identified 18 genes that were differently expressed between both stages, finding again MMP11 and UBE2C upregulated in invasive carcinomas.

In order to find out if these proteins could have a role in breast tumorigenesis progression, we studied if they are differentially expressed between matched in situ and invasive carcinomas in histological sections where we can assess their subcellular localization, by immunohistochemistry.

2. RESULTS

IHC quantification for MMP11, SYTV, ADM and UBE2C was performed on each set of the 22 TMA slides, using patient-matched DCIS/IDC tumor samples. Data on ER, PgR, HER2, P-cad, CK5, EGFR, Ki-67 status, histological grade and lymph node metastases were already available and published for this series [208].

EVALUATION IN NORMAL BREAST

In normal breast tissue, it can be observed that MMP11, SYTV, ADM and UBE2C expression was absent, as observed in Figure 1.

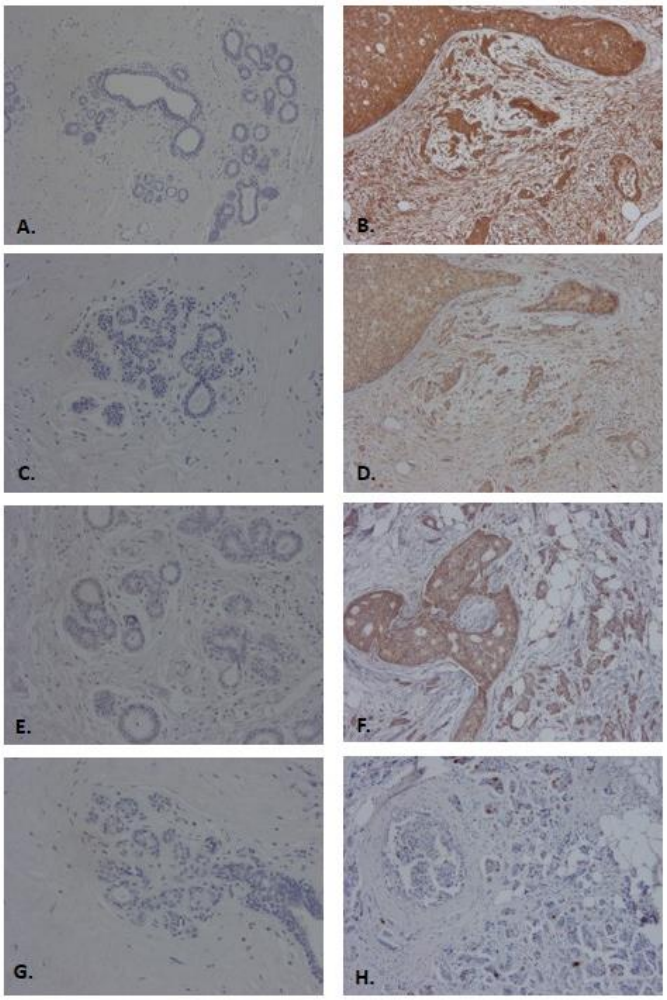


Figure 1: Expression of proteins studied by IHC on TMAs. **A.** and **B.** represent MMP-11expression in normal breast tissue and *in situ* and invasive breast carcinoma, respectively, 100x; **C.** shows Synaptotagmin V expression in normal breast and **D.** in *in situ* and invasive counterparts, 100x; **E.** represents Adrenomedullin

expression in normal and **F.** in *in situ* and invasive components, 100x; **G.** and **H.** shows UBE2C expression in normal and DCIS and IDC, respectively, 100x

EVALUATION OF THE IN SITU COMPONENT

The evaluation of the DCIS component, in each sample, revealed that , only 9 cases (5%) had absent expression of MMP11, whereas 34 cases (18%) had weak expression, 50 cases (26%) showed intense expression and the majority of the cases (96 cases, 51%) had moderate expression. Regarding the expression of SYTV, 20 cases (10%) showed no reactivity for the marker, 62 cases (33%) showed weak expression, 72 cases (38%) had moderate expression and 35 cases (19%) had intense expression. For ADM, the majority of the cases (121 cases, 66%) were negative, whereas 22 cases (12%) showed weak expression, 30 cases (16%) had moderate expression and only 10 cases (5%) showed intense expression. Concerning UBE2C, the majority of the cases (101 cases, 53%) were positive, whereas 88 cases (47%) were negative.

EVALUATION OF THE INVASIVE COMPONENT

Regarding the invasive counterpart, we could find that 10 cases (5%) showed no reactivity for MMP11, 47 (25%) cases showed weak expression, 81 cases (43%) presented moderate expression and 51 cases (27%) showed intense expression. For SYTV expression, 21 cases (11%) had absent expression, whereas 72 and 62 cases (38% and 33%) showed weak and moderate expression respectively, and 34 cases (18%) showed strong expression. Concerning the expression of ADM, the majority of the cases (127 cases, 69%) were negative, 22 cases (12%) showed weak expression, 25 cases (14%) had moderate expression and only 10 cases (5%) showed intense expression. Concerning UBE2C, the majority of the cases (118 cases, 62%) were negative, whereas 71 cases (38%) were positive for this protein.

COMBINED EVALUATION OF THE *IN SITU* AND INVASIVE COUNTERPARTS IN THE SAME PATIENT

The analysis of MMP11 expression in the transition from the *in situ* to the invasive counterpart (Fig. 1A) showed that the majority of the cases (85%) maintained the

expression for this marker, whereas only 29 cases showed a differential expression for this marker. Among the differences, the majority of cases showed loss of expression in the invasive counterpart. Accordingly, we found that 15 cases classified as moderate expression in the *in situ* component, but as weak expression in the invasive counterpart. In contrast, 10 cases showed gain of MMP11 expression in the invasive tumor fraction.

Regarding the expression of SYT V (Fig. 1B), only 22 cases showed different expression between both components, meaning that 88% of the cases maintain the expression of this marker. The main differences were the loss of SYT5 expression in 14 cases, whereas 8 cases gained its expression during cancer progression.

Analyzing individually each case for ADM expression (Fig. 1C), we could show that 38 cases showed a distinct expression between the two components: 22 cases showed loss of expression and 16 cases showed gain of expression. 79% of the cases maintained ADM expression between *in situ* and invasive components.

The major alterations were found for UBE2C expression between matched *in situ* and invasive tumor parts (Fig. 1D) of breast carcinomas. Although the percentage of concordance in the expression of UBE2C was around 75%, 47 cases showed different profiles for UBEC2 between both components. 40 cases showed loss of expression in the invasive counterpart, whereas only 7 cases showed the gain of expression. The average of UBE2C expression in the *in situ* component was 53.4%, whereas it decreased for 37.5% in the invasive tumor fraction.

Concerning the associations found between the proteins studied, we could only describe a statistically significant association between HER-2 and UBE2C expression in the *in situ* component ($p=0.001$). No other significant associations were obtained between the markers studied and clinical-pathological features of the tumor series (Table 1). Figure 2 shows an immunohistochemistry array of the proteins studied, representing the gains and losses in the progression from *in situ* to invasive breast carcinoma.

Table 1: Associations between UBE2C with some biomarkers expression and clinical-pathological parameters

		UBE2C					
		In Situ		p value	Invasive		p value
		Positive n(%)	Negative n(%)		Positive n(%)	Negative n(%)	
ER	Positive	74 (52%)	68 (48%)	0.52	54 (39%)	85 (61%)	0.54
	Negative	27 (57%)	20 (43%)		17(34%)	33 (66%)	
PgR	Positive	50 (51%)	48 (49%)	0.48	28 (30%)	66 (70%)	0.20
	Negative	51 (56%)	40 (44%)		43(45%)	52 (55%)	
HER2	Positive	8(9%)	86 (91%)	<0.001	19 (51%)	18 (49%)	0.001
	Negative	30 (32%)	65 (68%)		52 (34%)	100 (66%)	
EGFR	Positive	4 (67%)	2 (33%)	0.50	4 (67%)	2 (33%)	0.13
	Negative	97 (53%)	86 (47%)		67 (37%)	116 (63%)	
CK5	Positive	10 (67%)	5 (33%)	0.28	7 (55%)	5 (42%)	0.12
	Negative	91 (52%)	83 (48%)		64 (36%)	113 (64%)	
P-Cad	Positive	15 (42%)	21 (58%)	0.11	13 (35%)	24 (64%)	0.73
	Negative	86 (56%)	67 (44%)		58 (38%)	94 (62%)	
Ki67	Positive	8 (53%)	7 (47%)	0.99	14 (52%)	13 (48%)	0.09
	Negative	93 (53%)	81 (47%)		57 (35%)	105 (65%)	
Histological Grade	L/I	30 (45%)	37 (55%)	0.07	20 (27%)	55 (73%)	0.05
	I/II	30 (45%)	37 (55%)		22 (41%)	32 (59%)	
	H/III	44 (69%)	20 (31%)		29 (48%)	31 (52%)	
Ganglionar Invasion	Present				26 (32%)	54 (68%)	0.21
	Absent				22 (43%)	29 (57%)	

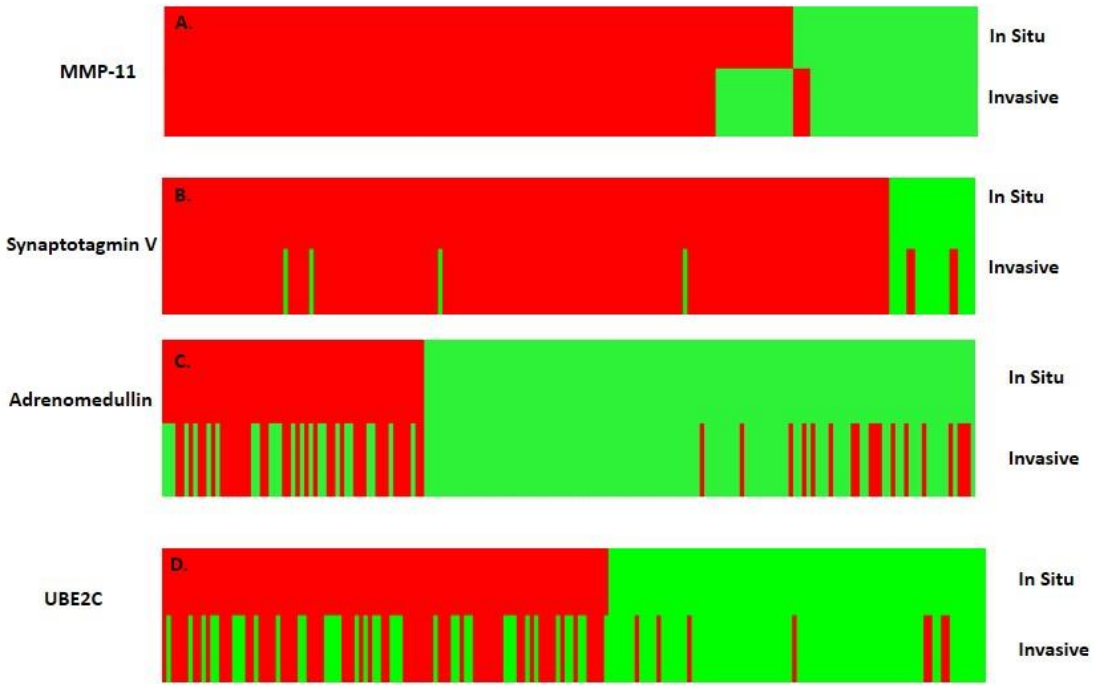


Figure 2: Immunohistochemistry array showing protein expression levels of MMP11 (A.), Synaptotagmin V (B.), Adrenomedullin (C.) and UBE2C (D.) in the progression from *in situ* to invasive carcinomas. Cases are arranged along the X-axis and proteins are arranged along the Y-axis. Within the heat map, red represents gain of expression whereas green represents loss of expression.

3. DISCUSSION

It is of great interest to understand the transcriptional program that drives invasive growth and several studies have been trying to identify genes that would mark the transition from *in situ* neoplastic cells to migrating invasive cells. Hannemann et a.l [120] identified a gene expression classifier of 35 genes which were differently expressed between the *in situ* and invasive breast cancer components.

Among these genes, MMP11, a protein of the matrix metalloproteinase family, involved in the breakdown of the extracellular matrix in both physiological and disease processes, has been described as been upregulated in invasive breast carcinomas [213], compared to normal breast tissue [214]. Ma et al. [146] also showed a significant increased expression of MMP11 in invasive carcinoma-associated stroma. MMP11 has also recently been shown to exhibit protease activity [215] and to promote tumor progression [216]. Schuetz and colleagues profiled the epithelium of patient-matched DCIS and IDC and found MMP11 to be upregulated in IDC relative to DCIS [131]. In the study of Hannemann and colleagues [120], MMP11 was also identified as able to distinguish IDC from DCIS. In our series, the expression of MMP11 was similar in *in situ* and invasive breast components, suggesting that the expression of MMP11 is needed for tumor cells already in DCIS stage.

Synaptotagmins are a family of type 1 membrane proteins that function as calcium sensors for the regulated exocytosis of neurotransmitters, neuropeptides and hormones [217]. Although a previous study [217] has showed an increase expression of this marker in invasive human breast cancer, we showed that the patterns of expression were similar between in *situ* and invasive breast carcinomas harboring in the same patient, suggesting that an increased expression may be observed in preinvasive and invasive stages, comparing to normal breast tissue.

ADM is also is expressed in a variety of tumors, including breast, endometrial and prostate cancer. ADM has been shown to be a mitogenic factor capable of stimulating growth of several cancer cell types, being described as upregulated in invasive breast carcinomas, associated with lymph node metastasis and increased tumor size [193]. Hsieh et al. [218] suggested that elevated levels of this protein were significantly associated with increased expression of potential downstream targets, such as apoptosis inhibitors, cell cycle regulators and inducers of tumor angiogenesis. However, our results showed that the percentage of expression in both *in situ* and invasive components were highly similar, with a slightly increase of invasive tumors expressing intense expression of

ADM. Since the percentage of concordance was 79%, ADM expression may be also acquired in the DCIS stage.

UBEC2 is an ubiquitin-conjugating enzyme composing the ubiquitin-proteasome system, which is required for the destruction of mitotic cyclins and for cell cycle progression. Furthermore, in breast cancer, an increased expression of UBE2C was associated with high tumor grade and cancer progression [130]. UBE2C also belongs to proliferative genes, which are known to constitute the majority of genes included in prognostic gene-expression signatures [219]. However, we could not find any correlation with genes involved in proliferation, such as Ki67; but, concerning the expression of UBE2C in *in situ* and invasive breast carcinomas, we observed an increased expression in DCIS tumors and a loss of UBE2C expression in the invasive counterpart. Loussouarn et al. have also described an upregulation of UBE2C in carcinomas comparing to normal breast tissue [220].

In general, among the losses and gains of the markers studied, the alterations in UBE2C were the most consistent ones: 40 cases showed loss of expression from DCIS to invasive breast carcinoma, whereas only 7 cases showed a gain of expression. The average of UBE2C was higher in the *in situ* component (53.4%) than in the invasive counterpart (37.5%), suggesting that maybe neoplastic cells require the expression of UBE2C to disrupt the basal membrane and to invade.

The only significant association found was between the expression of HER2 and UBE2C the *in situ* component only. However, as HER2 overexpression has been previously described as associated with more rapid progression to invasive disease [221], the association that we obtained could be mimicked by the overexpression of HER2 in DCIS tumors [222]. These results can also raise the hypothesis that the subgroup of DCIS tumors that loss the expression of UBE2C in the invasive counterpart may progress more rapidly. On the other hand, recently a study [128] focusing the alterations in the progression from *in situ* to invasive breast cancer, suggests a bottleneck effect during the progression, where only the subclones harboring a specific repertoire of genetic aberrations are selected and pass through the evolutionary bottleneck, maybe explaining why some genetic alterations and consequently differences in protein level such as UBE2C expression could occur during the progression from *in situ* to invasive breast cancer. The absence of differences in MMP11, SYTV and ADM can occur due a convergent phenotype [128], where several combinations of somatic genetic or epigenetic aberrations result in the acquisition of the biological properties required for cancer cells to progress *in situ* to invasive disease.

Based on the above, we have shown that, by immunohistochemistry, the expression of these four markers (MMP11, SYTV, ADM and UBEC2) did not differ

significantly between DCIS and IDC. Thus, we were not able to validate the set of specific candidate genes that were previously identified by microarray analysis, supporting that probably the molecular alterations driving invasion occur prior to the morphological modifications of the lesions, explaining as well as that alterations are mainly found when normal breast tissue and the invasive one are compared.

CHAPTER VI

THE IMPORTANCE OF METABOLIC ALTERATIONS IN THE PROGRESSION FROM *IN SITU* TO INVASIVE BREAST CARCINOMAS: THE ROLE OF CAV-1 AND MCT4 IN BREAST TUMORIGENESIS

PAPERS RELATED TO THIS CHAPTER:

Martins D, Beça FF, Sousa B, Baltazar F, Paredes J, Schmitt F. Loss of Caveolin-1 and gain of MCT4 expression in the tumor stroma: key events in the progression from an in situ to invasive breast carcinoma. Cell Cycle. 2013 Aug 15; 12 (16): 2684-90.

1. INTRODUCTION

Breast cancer is a heterogeneous and complex disease, encompassing a variety of pathological entities with distinct clinical behaviors. The development of new technologies has offered the opportunity to explore the molecular complexity of human breast carcinomas [144]. However, despite these advances, the mechanisms controlling the transition from an *in situ* to an invasive carcinoma still remain unclear. Therefore, there is a significant interest in identifying molecular events driving invasive progression, not only to determine at which point the lesion is most likely to progress to malignancy, but also to identify new molecular targets that could trigger the progression at early stages [144]. Several studies have evaluated the gene expression profiles of both ductal carcinomas *in situ* (DCIS) and invasive ductal carcinomas (IDC) [120, 130-132, 147, 199, 209], but only few compared the *in situ* and invasive components within the same breast tumor [120, 130-132]. In fact, although some genes have been described as differentially expressed between *in situ* and invasive components, the majority of the studies failed to demonstrate significant differences between the expression of the codified proteins in the neoplastic epithelial cells of DCIS and IDC [131, 223]. Recently, our group, using patient-matched DCIS/IDC tumor samples, showed concordance between *in situ* and invasive molecular profiles in 94% of the cases [208]. These results suggested that the alterations in the tumor microenvironment would have a more important role in the progression from an *in situ* to an invasive phenotype than the biology of the tumor cells *per se*, which showed a tendency to be maintained between these both components.

Actually, it is widely accepted that any cancer is a complex system composed not only by neoplastic cells but also by a fine-tuned microenvironment. The first reference to the importance of the microenvironment in cancer comes from Paget, with his proposal of the “seed and soil” hypothesis. Unexpectedly, this concept was “forgotten” and only “recovered” several years later. In breast cancer, tumor microenvironment plays a key role in defining tumor behaviour and patient outcome [158]. Gene expression changes that occur in cancer-associated stroma are known to be implicated in prognosis, as well as in cancer progression [146, 167, 207]. Ma and colleagues, using gene expression profiling, provided strong evidence that the stroma co-evolves with the epithelial compartments during cancer progression [146]. Analyzing 14 patients with matched normal epithelium, normal stroma, tumor epithelium and tumor-associated stroma, the authors proposed that microenvironment participates in tumorigenesis even before tumor cells invade the stroma and it may play an important role in the transition from preinvasive to invasive growth[146].

Caveolin-1 (Cav-1), a scaffolding protein mainly involved in vesicular transport, cholesterol homeostasis and signal transduction, has been associated to the progression from *in situ* to invasive carcinoma [194, 224]. Lisanti and colleagues showed that Cav-1 loss in tumor stroma was associated with an increased risk for early recurrence, metastasis and decreased overall survival in breast cancer, being also a strong prognostic factor for basal-like breast carcinomas [225, 226]. In DCIS, a loss of stromal Cav-1 was predictive of disease recurrence and progression to invasive cancer, since all the patients with loss of Cav-1 recurred and 80% of them progressed to invasive disease[194]. Moreover, loss of stromal Cav-1 has been related with stromal MCT4 expression in triple-negative breast cancers, also predicting for poor clinical outcome [195]. This protein is a major transporter directly responsible for L-lactate efflux from glycolytic cells and a functional marker of oxidative stress and hypoxia [227]. In addition, it seems to have a role in stromal breast cancer metabolism, since it has been demonstrated that breast cancer cells induce MCT4 overexpression in stromal fibroblasts [196].

Since stromal expression of MCT4 and the association between Cav-1 and MCT4 had never been implicated in the progression from DCIS to IDC, the aim of this study was to better understand the stromal interactions surrounding *in situ* and invasive components of breast carcinomas, evaluating the stromal expression of Cav-1 and MCT4, using patient-matched DCIS/IDC tumor samples.

2. RESULTS

CAV-1 AND MCT4 EXPRESSION IN NORMAL BREAST

In normal breast, it can be observed that Cav-1 expression was absent from the epithelium, whereas it was observed expression in the stromal component, as previously described [194, 225, 226]. MCT4 expression was absent in both epithelial and stromal components, as observed in Figure 1A.

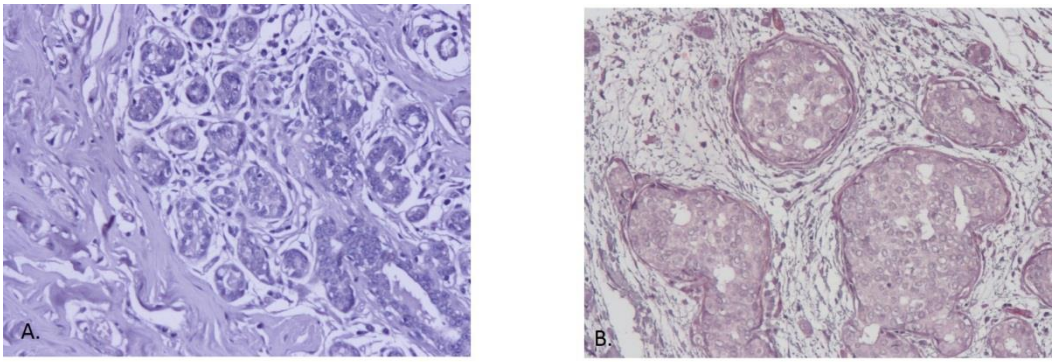


Figure 1: IHC expression of stromal MCT4 in normal and *in situ* component. It can be observed absent stromal MCT4 expression in normal breast (A) and in in situ component (B), 200x.

STROMAL CAV-1 EXPRESSION IN THE PROGRESSION FROM *IN SITU* TO INVASIVE CARCINOMA

In the DCIS component, only 19 cases (13%) showed no Cav-1 expression in the stroma, whereas 55 cases (39%) had moderate expression, and the majority had strong expression of stromal Cav-1 (67 cases - 48%). Analyzing the association between the histological grade of the *in situ* component and the expression of Cav-1, it was observed that the majority of the cases were high grade DCIS tumors (35,5%) and among them 56% showed strong expression for Cav-1 (p=0,04).

In the invasive component, the majority (n=108, 76%) of the cases showed absent Cav-1 expression in the stroma, with only 27 cases (19%) with moderate expression and 7 cases (5%) with strong expression. The association between the histological grade and the expression of Cav-1 is lost in invasive counterpart due to the majority of the cases have shown absent expression of the stromal marker. Figure 2 represents the expression levels of stromal Cav-1 in *in situ* and invasive components, where it can be observed a significant decrease of Cav-1 from DCIS to IDC. An IHC example of Cav-1 in *in situ* and invasive components is shown in Figure 3**.**

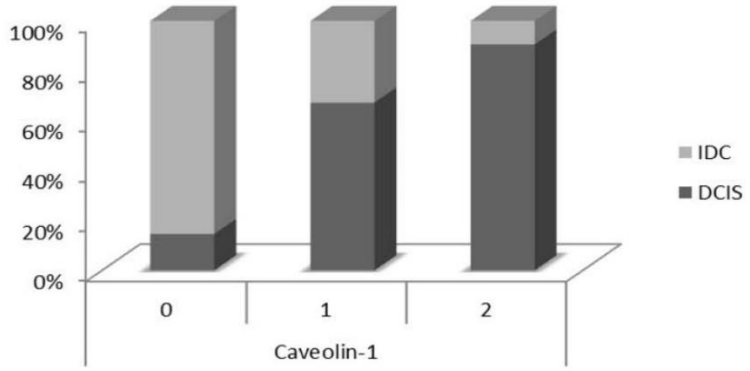


Figure 2: Expression levels of stromal Cav-1 in *in situ* and invasive components of breast carcinomas. It is possible to notice a significant decrease of Cav-1 stromal expression from DCIS to IDC.

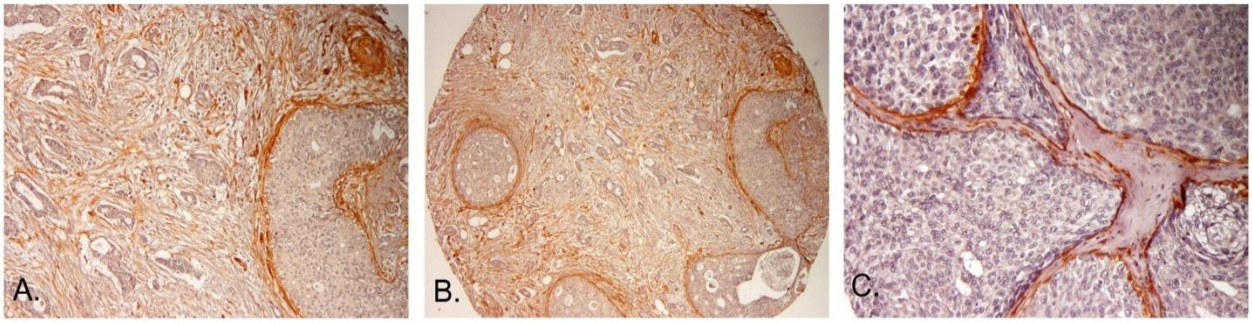


Figure 3: IHC expression of stromal Cav-1 in *in situ* and invasive components. Note the strong expression of Cav-1 in DCIS, from low (A and B, 100x and 200x respectively) to higher magnification (C, 400x).

Regarding the progression from *in situ* to invasive carcinoma, analyzing each case for both matched components, 106 cases (75%) showed loss of stromal Cav-1 expression, whereas 35 (25%) cases maintained protein expression. None of the cases showed gain of stromal Cav-1 expression.

STROMAL MCT4 EXPRESSION IN THE PROGRESSION FROM *IN SITU* TO INVASIVE CARCINOMA

Considering the DCIS component, the majority of the cases were negative (n=131; 93%), 10 cases (7%) showed moderate expression and 5 cases (3%) were classified as strong for stromal MCT4. In the invasive component, it was observed a strong expression of MCT4 in the stroma of the majority of the cases (n=73; 50%), whereas moderate expression was observed in 63 (43%) cases; in the remaining 11 cases (7%), no expression of stromal MCT4 was observed. In figure 1B, note the absence expression of

MCT4 surrounding DCIS component. Analyzing the histological grade in these cases, it was not observed any association between the expression of MCT4 and the grade of the tumors.

Figure 4 depicts the expression levels of stromal MCT4 *in situ* and invasive components, showing an increased expression of stromal MCT4 in the invasive component. Figure 5 represents by IHC the strong MCT4 stromal expression in invasive component. Concerning the transition from *in situ* to invasive carcinoma, in terms of gains and losses of MCT4 in the stroma, we found that 126 cases (87%) gained expression in the invasive component, 19 cases (13%) maintained and none loose the expression.

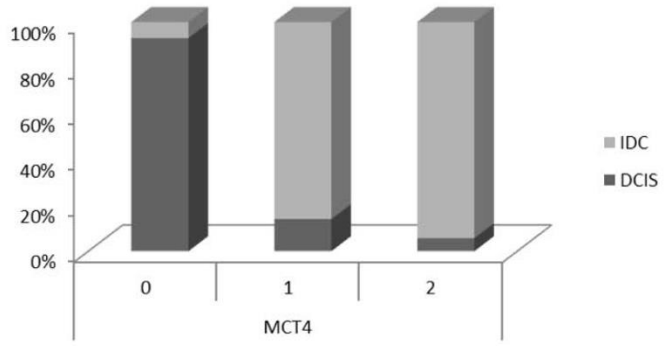


Figure 4: Expression levels of stromal MCT4 in *in situ* and invasive carcinomas**.** There is a significant increased expression of stromal MCT4 in the invasive component of breast carcinomas, when compared with DCIS.

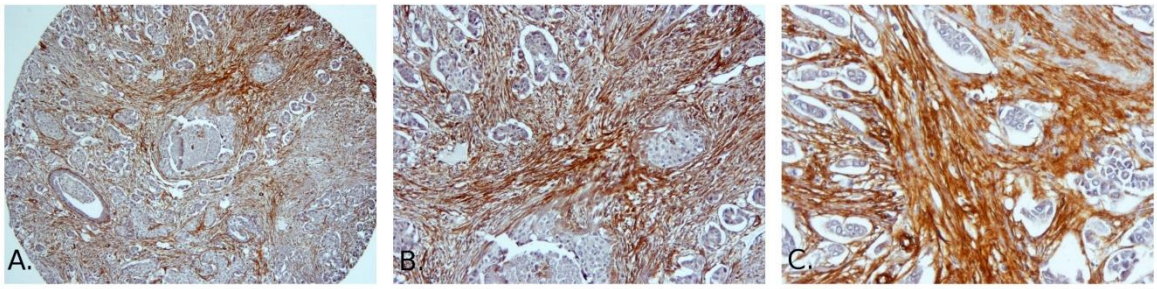


Figure 5: IHC expression of stromal MCT4 in *in situ* and invasive components. Note the strong MCT4 stromal expression in invasive component, from low (A and B, 100x and 200x, respectively) to high magnification (C, 400x).

COMBINING STROMAL CAV-1/MCT4 IN THE PROGRESSION FROM *IN SITU* TO INVASIVE CARCINOMA

Analyzing matched *in situ* and invasive components for stromal expression of Cav-1 and MCT4 (Table 1), it was possible to observe a statistically significant association between the loss of stromal Cav-1 and the concomitant gain of MCT4 in the same case ($p<0.0001$). Interestingly, 75% of the cases that loss Cav-1 stromal expression in the transition from *in situ* to invasive cancer, also gained MCT4 expression in the stroma. There were only 4 cases (3%) with loss of Cav-1 in the stroma that maintained MCT4 expression, and 16 cases (12.5%) that gained MCT4 and maintained Cav-1 stromal expression. In 12 cases (10%), there was the maintenance of stromal expression for both markers. Figure 6 represents an IHC array with the expression levels of these proteins in the progression from *in situ* to invasive carcinoma.

Table: Association between stromal Cav-1 and MCT4 expression levels in the transition from in situ to invasive breast carcinoma

		MCT4 (*In Situ* to Invasive)		
		Loss of expression N (%)	Maintenance of expression N (%)	Gain of expression N (%)
Cav-1 (In Situ to Invasive)	Loss of expression N (%)	0 (0%)	4 (3%)	94 (75%)
	Maintenance of expression N (%)	0 (0%)	12 (10%)	16 (12.5%)
	Gain of expression N (%)	0 (0%)	0 (0%)	0 (0%)

p value: ≤0.001

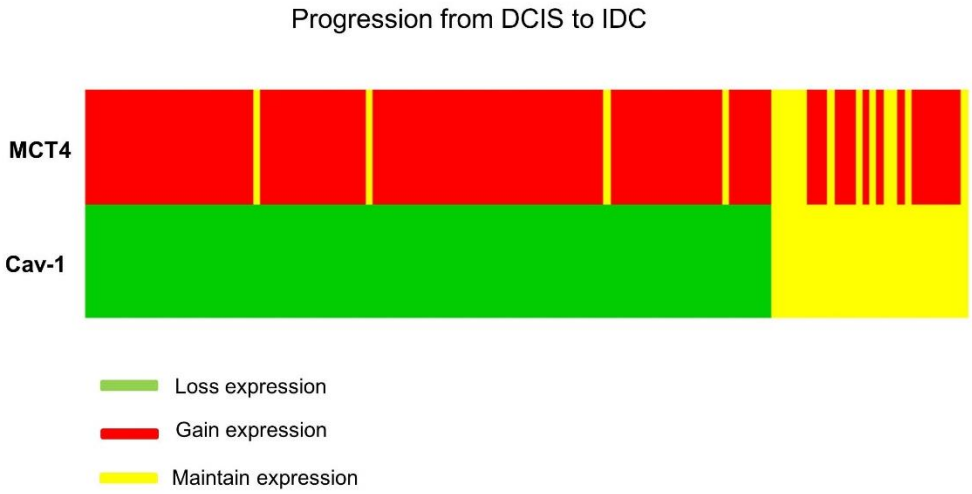


Figure 6: Immunohistochemistry array showing protein expression levels of stromal Cav-1 and MCT4 in the progression from *in situ* to invasive carcinomas. Cases are arranged along the X-axis and proteins are arranged along the Y-axis. Within the heat map, red represents gain of expression, green represents loss of expression and yellow represents maintained expression from in situ to invasive carcinoma within the same case.

3. DISCUSSION

The mechanisms that mediate the progression from DCIS to IDC in the breast are still largely unknown. However, it is now widely acknowledged that accumulation of genetic anomalies contributes to the acquisition of an increasingly aggressive, invasive or therapy-resistant tumor phenotype [144]. Nevertheless this knowledge did not improve the predictive power of standard pathological parameters for breast cancer, nor explained the mechanisms of invasiveness.

Cav-1 plays an important role in tumor stroma and recent studies demonstrate that the loss of stromal Cav-1 is associated with advanced tumor and nodal stage, lymphovascular invasion, metastasis, early recurrence, tamoxifen resistance and reduced progression-free survival in invasive breast cancer [174, 228, 229] Additionally, loss of stromal Cav-1 also has prognostic value in a particularly aggressive subgroup of breast cancers, namely the triple-negative and basal-like breast carcinomas, whereas high levels of this protein were correlated with reduced tumor size, low grade, reduced metastasis and improved survival [226, 229, 230].

Interestingly, loss of stromal Cav-1 also predicts for recurrence and early disease progression in DCIS patients. Witkiewicz *et al.* reported that 80% of the DCIS patients, which underwent surgical excision and recurred with invasive breast cancers, showed reduced or absent levels of stromal Cav-1 in these tumors [194]. In our series, using patient-matched DCIS/IDC tumor samples, it was observed that the majority of the cases showed strong expression of Cav-1 expression in the stroma of DCIS, whereas 76% of the cases showed absent expression for this marker in the stroma of the invasive counterpart. Thus, regarding the progression to invasiveness, it seems that the loss of Cav-1 expression in the stroma is important for tumor invasion.

Actually, it has been already described that loss of Cav-1 in stromal cells may also increase angiogenesis and tumor growth [224]. Goetz *et al.* demonstrated that *in vivo* and *in vitro* expression of Cav-1 in cancer-associated fibroblasts facilitates tumor cells invasion and accelerate the *in vitro* proliferation and *in vivo* tumorigenesis [231, 232].

Recent data reveals that loss of Cav-1 induces a metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells - the “reverse Warburg effect”, where cancer cells induce upregulation of multiple glycolytic enzymes in neighbouring stromal fibroblasts [174, 233, 234]. Cav-1 is degraded resulting in a loss of stromal Cav-1 expression [195]. At the same time, the breast cancer cells induce MCT4 overexpression in stromal fibroblasts [195].

MCT4 is a monocarboxylate transporter that functions as a shuttle to extrude L-lactate from cells using aerobic glycolysis for energy metabolism [227]. Although the transporter role of MCT4 has been widely accepted in cancer epithelium, the prognostic value of MCT4 expression is highly compartment-specific and restricted to the tumor stroma, being high stromal MCT4 levels associated to poor patient overall survival [196, 235, 236]. In our series, analyzing DCIS and IDC separately, it was observed an increase of MCT4 expression, since in DCIS the majority of the cases were negative, whereas, in the invasive counterpart, 50% of the cases showed strong expression for MCT4. Considering the progression from *in situ* to invasive breast carcinoma, using matched DCIS/IDC tumor samples, 87% cases gained MCT4 expression, whereas none showed loss of expression, suggesting that the gain of stroma MCT4 provides evidence for the existence of a stromal-epithelial lactate shuttle which fuels the tumor growth [196].

Regarding the relation between MCT4 and Cav-1 expression, Witkiewicz *et al.* [195] using 164 invasive breast cancer samples, verified that stromal MCT4 and stromal Cav-1 levels were inversely related, being high levels of stromal MCT4 directly correlated with a loss of stromal Cav-1 immunostaining [195]. Most notably, cases with absent stromal Cav-1 are most likely to present strong stromal staining for MCT4 and, in contrast, cases with strong expression for Cav-1 are most likely to be stromal MCT4 absent.

Nevertheless, studies regarding the role of Cav-1 and MCT4 in the transition from *in situ* to invasive breast carcinoma were still lacking. In our series, using matched DCIS/IDC and analyzing the concomitant expression of stromal Cav-1 and MCT4, 75% of the cases showed loss of Cav-1 with simultaneously gain of MCT4 in the stroma, suggesting that these events are important for tumor cells to progress and invade.

Our results are explained by the recently “Two-Compartment Tumor Metabolism” model and the “Reverse Warburg Effect”, suggesting that the loss of Cav-1 causes the metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells [174]. In figure 7, a hypothetical model summarizing he alterations in Cav-1 and MCT4 in the stroma of matched *in situ* and invasive breast carcinoma is shown. The oxidative stress promoted by the tumor cells induces autophagy in cancer associated fibroblasts (CAFS) that degrade Cav-1 in the *in situ* stromal compartment and also secrete energy-rich metabolites, such as L-lactate, ketone bodies and pyruvate as a consequence of metabolic alterations. During the progression do invasive carcinoma, the loss of Cav-1 induces MCT4 expression due to the amount of energy metabolites, used to promote cancer cell glycolysis, aggressive tumor growth and ultimately invasion of breast cancer cells.

Many of the cited studies quantify one or both markers in breast cancer stroma. However one potential limitation of the quantification methodologies used is the lack of a clear and

reproducible definition of stroma, especially regarding DCIS cases. In our case, since all IHC scoring was performed by the same experienced pathologist, we consider this does not affect internal validity and therefore does not affect the results obtained and conclusions drawn.

In summary, it was shown that the loss of stromal Cav-1 and the concomitant gain of stromal MCT4 have a putative role in the transition from *in situ* to invasive carcinoma of the breast. Therefore, we propose that Cav-1 and MCT4 may represent valuable biomarkers for breast cancer progression. Thus, determining the nature of the cooperation between tumor cells and the microenvironment that leads to invasion could identify therapeutic strategies to prevent the transition from *in situ* to invasive breast carcinoma.

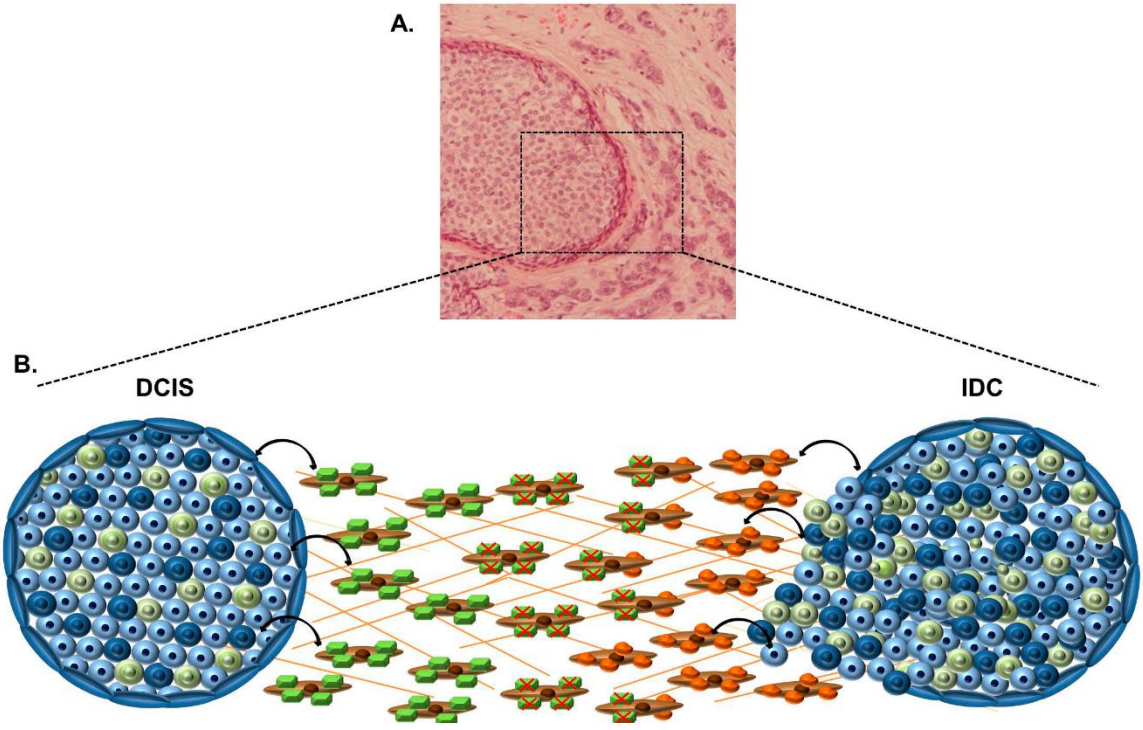


Figure 7: Alterations in Cav-1 and MCT4in the stroma of matched *in situ* and invasive breast carcinoma. A. H&E stained tissue section of human breast cancer, showing *in situ* and invasive components of breast carcinoma (100x). B. Hypothetical model summarizing the importance of Cav-1 and MCT4 in the progression from DCIS to IDC. During the progression to invasive carcinoma, Cav-1 is degraded by oxidative stress-induced autophagy in cancer-associated fibroblasts, resulting in a loss of Cav-1. At the same time, the loss of Cav-1 induces a metabolic reprogramming of stromal cells where cancer cells induce upregulation of MCT4 by stromal fibroblasts, in invasive counterpart.

General Discussion

GENERAL DISCUSSION

The work presented throughout this thesis addressed one major topic in breast cancer research, which is the progression from *in situ* to invasive breast carcinoma. Herein, we highlight the relevance of both epithelial tumor cells in the progression and additionally provide innovative data concerning the microenvironment surrounding the tumor cells. We explore the role of the epithelial tumor cells, pushing the borders of the knowledge about the molecular profiles of *in situ* and invasive carcinomas and study if previously described genes could effectively discriminate between both components. On the other hand, we also explore the role of stromal components in breast tumorigenesis, focusing our attention in the dynamic cross-talk interactions between the stroma and the cancer cells, both in *in situ* and invasive components.

The majority of the studies that explore the mechanisms underlying breast tumorigenesis are based in independent series of *in situ* and invasive breast carcinomas in order to find the molecular alterations that could trigger the progression. Although we understand that the identification of specific gene expression patterns of DCIS and IDC separately could help to elucidate the mechanisms underlying the evolution from *in situ* to invasive breast cancers, this model does not represent a basis of progression to invasive tumors, due to the lack of the matched counterpart. The great advantage of our series of 189 breast carcinomas relies precisely on the existence of *in situ* and invasive components in the same sample.

Based on the above, and aiming a deeper insight into the molecular profiles of *in situ* and invasive breast carcinomas, both components were classified into the different subtypes of breast carcinomas: Luminal A, B, HER2 overexpressing and Basal-like breast carcinomas in an attempt to translate the molecular classification, using immunohistochemistry and tissue microarrays. The work showed that there were no significant differences in molecular classifications of concomitant *in situ* and invasive tumors, as in 94% of the cases the molecular classification was identical between *in situ* and invasive components, suggesting that these tumors are not present as different entities belonging to different molecular profiles, but rather belong to the same molecular subtype. In fact, the different molecular subtypes have different progression forms, evolving low grade *in situ* tumors to low grade invasive tumors and high-grade *in situ* tumors to high grade invasive tumors. These results also support the theory of the parallel disease, which explains that in the progression of the majority of breast cancer cases there is a commitment of the *in situ* subtype carcinoma to a specific subtype of invasive carcinoma, contradicting the linear pattern supported by the theory of linear progression.

Understanding that effectively the molecular subtypes are similar in *in situ* and invasive components and knowing that the classical histopathology has a limited ability to predict which cases are in risk of developing an invasive disease, there is an immediate need to characterize new molecules that uncover the molecular biology of *in situ* carcinomas and its transition to invasive breast cancer, and understand if some genetic alteration could be able to trigger the transition from *in situ* neoplastic to migrating invasive cells. In the studies that focus their attention in the identification of novel molecular markers characterizing the transition, in order to avoid that the expression differences could be based on the genetic background of individual patients, DCIS and IDC were compared in a matched-pair analysis. Our aim was to found a set of genes that could be important in the progression from DCIS to IDC and to validate, by immunohistochemistry, if effectively these genes discriminate between the components. We compared gene expression data obtained from different studies and we gather four genes represented in the different genes lists, previously found to characterize the transition. It was not surprising that the genes were associated with important roles in cancer progression, such as epithelial-mesenchymal transition, invasion, cell adhesion and interaction, emphasizing the importance of changing intercellular network during the process of invasion. MMP11, Synaptotagmin V, Adrenomedullin and UBE2C were the set of genes selected in our studies and although all these genes have already been described as upregulated in the invasive component, any studies have showed by immunohistochemistry in a series of matched *in situ* and invasive components, if these genes were differently expressed. In our series, the expression of the markers did not differ significantly between DCIS and invasive breast cancers. We were not able to validate using immunohistochemistry, the set of specific candidate genes, namely MMP11, Adrenomedullin, Synaptotagmin V and UBE2C, previously found by RT- PCR, suggesting that probably the molecular alterations which drive invasion occur prior to the morphological modification of the lesion.

On the other hand, the understanding of tumor metabolism has evolved significantly over the last years. Only recently was recognized that there is a significant stromal-epithelial metabolic coupling and our study addresses the question of step-wise transformation from DCIS to IDC, comparing the stromal metabolism in regions affected by DCIS and IDC in the same patients. Cav-1 and MCT4 emerge as metabolic proteins, with Cav-1 involved mainly in vesicular transport, cholesterol homeostasis and signal transduction, and MCT4 as a marker of oxidative stress and hypoxia. Loss of Cav-1 is a marker of glycolitic metabolism, and it has been associated with high risk of progression from DCIS to IDC [194]. In our series, loss of Cav-1 was found in 75% in invasive carcinomas compared with the *in situ* counterpart, with no increase of Cav-1 expression in the any case during the progression from DCIS to IDC. Since high stromal MCT4

expression was already associated with loss of Cav-1 in triple negative breast cancers [195], we found that it was low in 90% of DCIS regions, whereas invasive carcinoma showed intense expression in 87% of the cases. Importantly, no decrease in MCT4 expression was found in the progression from DCIS to IDC in any case. Actually, it has been described that the direct contact between breast carcinoma cells and stromal cells is sufficient to induce loss of Cav-1 and gain of MCT4 expression in the stroma, increasing glycolysis and oxidative stress [196, 237]. Thus, stromal transformation may be a new hallmark of progression from DCIS to IDC and it will be important to evaluate inhibitors of stromal transformation in this context of breast cancer progression. On the other hand, metabolic reprogramming of the microenvironment is a key step in cancer progression and may be able to better predict which patients will develop IDC in the setting of DCIS, by characterizing stromal metabolism rather than features of carcinoma cells [238].

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Appendix

Publications

Publications

Paper I

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Original contribution

Molecular phenotypes of matched in situ and invasive components of breast carcinomas☆

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Summary The current system of pathologic classification of human breast cancers does not take into account the biologic determinants of prognosis, nor is there a consensus regarding the progression from in situ to invasive carcinoma. The present study compared the molecular phenotypes of in situ and invasive components of breast cancer in the same sample. We built a series of 189 in situ and invasive carcinomas using tissue microarrays and classified them according to their immunoprofiles regarding estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, epidermal growth factor receptor, cytokeratin 5, P-cadherin, and the antigen Ki-67 into luminal A and B, human epidermal growth factor receptor 2 overexpressing, and basal-like carcinomas. We also correlated the subgroups of carcinomas with some of the classical prognostic factors such as histologic grade, tumor size, and lymph node metastasis, as well as with the age of the patient at diagnosis. The overall concordance on the molecular phenotypes between in situ and invasive components was 94%. For the in situ component, 63% of the cases were luminal A; 15%, luminal B; 12%, human epidermal growth factor receptor 2 overexpressing; and 7%, basal-like. Regarding the invasive component, 61% of the cases were luminal A; 16%, luminal B; 12%, human epidermal growth factor receptor 2 overexpressing; and 8%, basal-like. The present study allowed the identification of different immunoprofiles of in situ and invasive breast carcinomas using a specific panel of biomarkers and showed that in most cases, there is a concordance between in situ and invasive component profiles, supporting the theory of parallel disease in breast tumorigenesis.

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1. Introduction

Breast cancer is the most common cancer in women, with more than 1 million cases occurring worldwide annually [1]. Despite significant diagnostic and therapeutic innovations, the effect on the mortality rate has been modest. One of the factors contributing to this limited success is the relative lack of understanding of the natural history of this disease [2]. For example, the transition from in situ to invasive carcinoma is still a poorly understood event [3].

Nowadays, it is widely stated that the natural history of breast cancer involves progression through clinical and pathologic stages [3,4] from premalignant hyperplastic breast lesions, with or without atypia, to carcinoma in situ and then invasive carcinoma [5-7]. On the basis of molecular, epidemiologic, and pathologic studies, ductal carcinoma in situ (DCIS) is thought to be a precursor of invasive ductal carcinoma [4]. Although this model is supported by clinical and molecular research [8-11], it is only a starting point to understand breast tumorigenesis, as the relation between preinvasive lesions and invasive carcinoma remains unclear [12]. From the available data, 2 models have been proposed recently to explain the transition from DCIS to invasive breast carcinoma (IBC). The first one, the *theory of linear progression* [5,7,13], suggests that low-grade DCIS progresses to high-grade DCIS and then to invasive ductal breast carcinoma. This model implies that tumor progression follows a linear pattern. However, there is evidence that some in situ carcinomas never progress to invasion and that some DCIS have more genetic alterations than some invasive carcinomas [14], a finding which does not fit in this multistep model. Consequently, a second model of breast cancer tumorigenesis has been proposed: the *theory of the parallel disease*, wherein low-grade DCIS tends to progress to low-grade invasive ductal breast cancer, whereas high-grade DCIS tends to progress to high-grade invasive breast cancer [12]. In this model, a specific subtype of DCIS matches a specific subtype of invasive breast cancer.

Gene expression profiling is known to be a powerful tool for identifying tumor molecular profiles and for correlating gene expression profiles with outcome in breast cancer [14]. In addition, it has been an important tool to explore the transcriptional program that leads to invasion, comparing in situ and invasive carcinomas. Recently, Dalgin et al [15] studied 36 breast cancer patients with different pathologic stages of disease and revealed a hierarchical portrait of breast cancer progression, identifying genes and pathways for each stage, grade, and molecular subtype. These authors suggested that the heterogeneity of the disease across molecular subtypes is higher than the heterogeneity of disease progression within a subtype, suggesting that tumors with different molecular profiles are in fact distinct diseases.

Several studies have concentrated on the identification of specific biomarkers that could define the subtypes of in situ and IBCs [16-18]. Our group and others demonstrated that it is possible to translate the molecular classification, using immunohistochemistry (IHC) and tissue microarrays (TMAs) [18], where estrogen and progesterone receptors (ER and PgR) and human epidermal growth factor receptor 2 (HER-2) expression identify luminal A and B and HER-2 overexpression subtypes, whereas tumor protein 63 (p63), cytokeratin 5 (CK5), and P-cadherin (P-cad) allow the identification of basal-like tumors [17]. Recently, Paredes et al [18] also demonstrated the importance of P-cad and CK5 as useful adjunct markers to distinguish the basal-like subtype among the in situ carcinomas.

However, it was never determined whether the in situ and invasive carcinomas that develop in a particular breast cancer patient belong to the same molecular subtype or are different entities belonging to different molecular profiles.

In this study, our aim was to compare the molecular phenotypes of in situ and invasive components of breast cancer in the same sample, using IHC and TMAs and a specific panel of biomarkers, previously described by our group [17,18].

2. Materials and methods

2.1. Tumor specimens

One hundred eighty-nine formalin-fixed, paraffin-embedded samples harboring in situ and IBCs in the same block were collected from the archives of the Pathology Institute of Araçatuba, São Paulo, Brazil (1996-2006). All cases were classified from hematoxylin and eosin (H&E)–stained sections. The DCIS samples were subdivided into 3 groups: low, intermediate, and high grade, according to the nuclear grade and the extent of necrosis, as previously published by our group [19]. Briefly, tumors harboring nuclear grade 3 were all considered high grade, whereas tumors with nuclear grade 1 or 2 with necrosis were considered intermediate grade, and those of nuclear grades 1 and 2 without necrosis were considered low grade. Invasive breast cancers were classified as grade I, II, or III according to the method described by Elston and Ellis [20]. Some relevant data were available for analysis, including age and clinicopathologic features such as tumor size and lymph node metastasis.

2.2. TMAs construction

Representative areas of the in situ and IBCs were selected on H&E-stained sections and marked on the corresponding paraffin blocks. Two 2-mm tissue cores were obtained from each selected specimen (donor block) and deposited in a paraffin (receptor) block using a TMA workstation (TMA Builder ab1802; Abcam, Cambridge, UK). Twenty-two TMA blocks were constructed, each containing 24 tissue cores (4 × 6). In each TMA block, nonneoplastic breast and liver tissue cores were included as a control and a TMA guide, respectively. After the construction, 2-μm tissue sections were cut and attached to Superfrost Plus glass slides.

An H&E-stained section from each TMA block was reviewed to confirm the presence of morphologically representative areas of the original lesions.

2.3. Immunohistochemistry

The sections were immunostained with primary monoclonal antibodies against ER, PgR, HER-2, epidermal growth factor receptor (EGFR), CK5, P-cad, and Ki-67. Immunostaining for ER, HER-2, and CK5 was performed using the streptavidin-biotin peroxidase technique (LabVision, Fremont, CA), whereas for PgR, EGFR, P-cad, and Ki-67, a horseradish peroxidase–labeled polymer (DakoCytomation, Carpinteria, CA) was used.

Antigen unmasking for ER, PgR, HER-2, and Ki-67 was carried out using 1:100 commercial citrate buffer, pH 6.0 (Vector Laboratories, Burlingame, CA) at 98°C, whereas a dilution of 1:10 from *tris*-ethylenediaminetetraacetic solution at pH 9.0 (DakoCytomation) was used for CK5 and P-cad. Epitope retrieval for EGFR was performed by proteolytic digestion (pepsin A, 4 g/L; Sigma-Aldrich, St. Louis, MO, USA) at 37°C.

The antigen retrieval time, antibodies, dilutions, and suppliers are listed in Table 1. After the antigen retrieval procedure, the slides were washed in phosphate-buffered saline and submitted to blockage of endogenous peroxidase activity by incubation of the slides in 3% hydrogen peroxide (Panreac, Barcelona, Spain) in methanol (Sigma-Aldrich). The slides were further incubated with blocking serum (LabVision Corporation kit) for 15 minutes and then incubated with the primary antibodies. After washes, the slides were incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (LabVision). Diaminobenzidine was used as a chromogen (DakoCytomation).

Table 1 Sources and dilutions of primary antibodies used in immunohistochemistry staining

Antibody	Clone	Manufacturer	Time of incubation (min)	Dilution	Antigen retrieval (min)
ER	SP1	Neomarkers (Fremont, CA, USA)	60	1:100	30
PgR	1A6	Novocastra (Newcastle, UK)	60	1:40	30
HER-2	SP3	Neomarkers	30	1:80	30
P-cad	56	Transduction Labs (Franklin Lakes, NJ, USA)	60	1:50	30
CK5	XM26	Neomarkers	60	1:50	30
EGFR	31G7	Zymed	60	1:100	30
Ki67	SP6	Neomarkers	60	1:300	30

For PgR, EGFR, P-cad, and Ki-67 staining, the secondary antibody was associated with horseradish peroxidase–labeled polymer (DakoCytomation) and immediately revealed with diaminobenzidine. Tissues were then counterstained with Mayer hematoxylin, dehydrated, and covered using a permanent mounting solution (Zymed, San Francisco, CA).

Positive controls were included in each run to guarantee the reliability of the assays. Nonneoplastic breast tissues, as well as normal breast surrounding the neoplastic cells, were considered internal controls.

2.4. Quantification of immunostaining

The IHC results were evaluated by 2 pathologists (F.S., F.M.). Both ER and PgR were examined for staining intensity, ranked from 1 to 3 (1, weak; 2, moderate; 3, strong) and extent, ranked from 1 to 10 (1, 0-10% cells; 2, 11%-20% cells; 3, 21%-30% cells; 4, 31%-40% cells; 5, 41%-50% cells; 6, 51%-60% cells; 7, 61%-70% cells; 8, 71%-80% cells; 9, 81%–90% cells; 10, 91%-100% cells) using the H-score method, which is used for other nuclear receptors as well [21,22]. The scores for intensity and extension were multiplied, and the cases were considered negative when the score was less than 4 and positive from 5 to 30. Concerning Ki-67, tumors with unequivocal nuclear staining in more than 14% of the cells were classified as highly proliferative, whereas tumors with less than 14% positive cells were considered to show low proliferation [23]. We considered positive the cases with membranous staining for P-cad and cytoplasmic staining for CK5 in at least 10% of the neoplastic cells. Expression of HER-2 was evaluated according to the DakoCytomation Hercept Test scoring system [24]. Cases were considered positive (overexpression) when immunostaining was classified as 3+. If a case is classified as 2+ by IHC, fluorescence in situ hybridization analysis was performed to determine if the tumor had *HER2* amplification. If amplification was confirmed, the tumor was classified as positive. If the tumor did not demonstrate amplification, it was considered negative. Staining for EGFR was also classified according to the Hercept Test scoring system. However, breast carcinomas were considered positive whenever the immunostaining was 2+ or 3+. Cases that were ER+ or PgR+ and HER-2 negative were classified as luminal A; cases ER+/PgR+ and HER-2+ or ER+ and with a high proliferative index (Ki-67+) were considered luminal B; ER− and PgR− and HER-2+ cases were classified as HER-2-overexpressing; cases that were negative for ER, PgR, and HER-2 and positive for EGFR or CK5 or P-cad were considered basal-like. Cases that lacked expression of all tested markers were considered "unclassified."

2.5. Statistical analysis

StatView 5.0 (SAS Institute Inc, Cary, NC) was used for statistical analysis. Univariate associations between ER, PgR, HER-2, EGFR, CK5, P-cad, KI67, tumor size,

Table 2 Frequencies of immunohistochemically defined subtypes of in situ and invasive breast cancers

Subtype	Frequency in situ component, n (%)	Frequency invasive component, n (%)
Luminal A	120/189 (63)	116/189 (61)
Luminal B	28/189 (15)	31/189 (16)
HER-2 overexpressing	23/189 (12)	23/189 (12)
Basal-like	13/189 (7)	14/189 (8)
Unclassified	5/189 (3)	5/189 (3)

histologic grade, and lymph node metastases in the presence of DCIS and invasive breast cancer were assessed using contingency tables and χ^2 tests. In all statistical analyses, $P \leq .05$ was considered significant.

3. Results

We performed IHC on each set of the 22 TMA slides for ER, PgR, HER-2, P-cad, CK5, EGFR, and Ki-67. Tables 2 and 3 summarize the clustering of a total of 189 immunohistochemically interpretable cases to allow sample characterization into 1 of the 5 previously described molecular subtypes. The molecular classification was made in an individual way for each of the tumor components (in situ and invasive) in the same block.

3.1. Evaluation of the in situ component

Among the in situ components, we observed that 63% of all tumors were considered luminal A, whereas the luminal B and HER-2–overexpressing subtypes comprised 15% and 12% of the cases, respectively. Basal-like tumors represented 7%, and the ones with null phenotype/unclassified were 3% (Table 2).

Because luminal cancer subtypes (A and B) were defined as positive for hormone receptors (ER, PgR), the percentage of cases positive for these 2 immunohistochemical markers was extremely high, as expected, with a higher prevalence for ER positivity (Table 3). For the luminal A subtype, 95% and 66% of the cases were ER and PgR positive, respectively, whereas for luminal B, 100% were positive for ER and 61% were positive for PgR. As initially defined, all luminal A tumors were negative for HER-2, and all luminal B lesions were positive for this marker. In the specimens negative for hormone receptors, all the cases overexpressing HER-2 were included in the HER-2–overexpressing cancer subtype, which are being the triple-negative ones (negative for ER, PgR, and HER-2) divided into basal-like or unclassified, according to the positivity for P-cad, CK5, and EGFR. Among the basal-like tumors, P-cad was the most prevalent marker, with 92% of the cases being positive, whereas only 23% and 38% of the cases were positive for EGFR and CK5, respectively.

Although basal markers are most commonly expressed in basal-like tumors, these can also be present in other cancer subtypes, if at a lower frequency. Concerning EGFR, although there were almost no positive cases in the luminal A and B subtypes, 10% of HER-2–overexpressing tumors also expressed EGFR. Also, CK5 was expressed by 17% of the HER-2–overexpressing in situ carcinomas, whereas only 3% and 7% of the tumors classified as luminal A or B, respectively, showed CK5 expression. Expression of P-cad also was common in HER-2–overexpressing tumors, which is being positive in almost half the cases (48%). Concerning the luminal cancer subtypes, P-cad expression was more abundant in luminal B (14%) than in luminal A (8%) lesions.

Concerning cell proliferation indexes, addressed by Ki-67 staining, basal-like tumors were the ones showing higher values (28%), followed by luminal B (19%). When we studied the association between the in situ histologic grade and molecular cancer subtypes (Fig. 1), we found that luminal A tumors were frequently classified as low grade (49%),

Table 3 Comparison of molecular subtypes and biomarkers for in situ and invasive components

		Luminal A		Luminal B		HER-2 overexpressing		Basal-like		*P*	
		In situ	Invasive	In situ	Invasive	In situ	Invasive	In situ	Invasive	In situ	Invasive
ER	+	114 (95%)	108 (93%)	28 (100%)	31 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	$P \leq .0001$	$P \leq .0001$
	−	6 (5%)	8 (7%)	0 (0%)	0 (0%)	23 (100%)	23 (100%)	13 (100%)	14 (100%)		
PR	+	79 (66%)	79 (68%)	17 (61%)	13(42%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	$P = .0002$	$P = .0001$
	−	41 (34%)	37 (32%)	11 (39%)	18 (58%)	23 (100%)	23 (100%)	13 (100%)	14 (100%)		
HER-2	+	0 (0%)	0 (0%)	15 (54%)	14 (45%)	23 (100%)	23 (100%)	0 (0%)	0 (0%)	$P \leq .0001$	$P \leq .0001$
	−	120 (100%)	116 (100%)	13 (46%)	17 (55%)	0 (0%)	0 (0%)	13 (100%)	14 (100%)		
EGFR	+	1 (1%)	1 (1%)	0 (0%)	0 (0%)	2 (10%)	2 (9%)	3 (23%)	3 (21%)	$P = .004$	$P = .001$
	−	119 (99%)	115 (99%)	28 (100%)	31 (100%)	21 (90%)	21 (91%)	10 (77%)	11 (79%)		
CK5	+	4 (3%)	2 (2%)	2 (7%)	0 (0%)	4 (17%)	5 (22%)	5 (38%)	5 (36%)	$P = .001$	$P \leq .0001$
	−	116 (97%)	114 (98%)	26 (93%)	31 (100%)	19 (83%)	18 (78%)	8 (62%)	9 (64%)		
P-cad	+	9 (7%)	9 (8%)	4 (14%)	4 (13%)	11 (48%)	11 (48%)	12 (92%)	13 (93%)	$P \leq .0001$	$P \leq .0001$
	−	111 (93%)	107 (92%)	24 (86%)	27 (87%)	12 (52%)	12 (52%)	1 (8%)	1 (7%)		

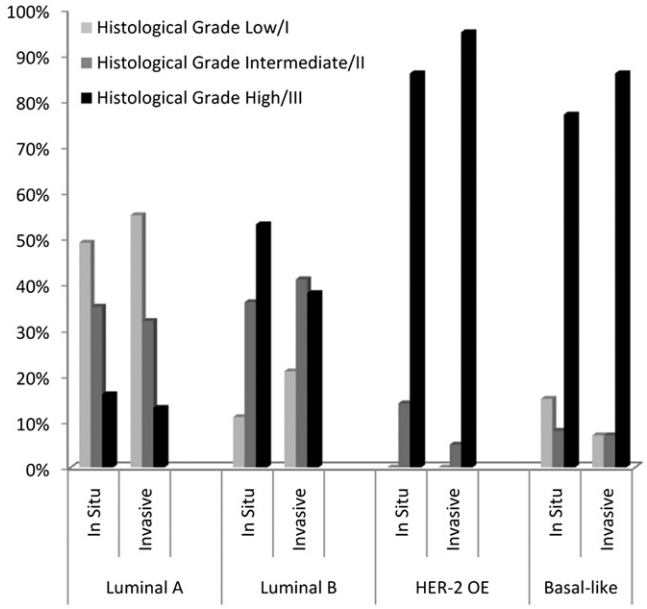

Fig. 1 Comparison of histologic grade among molecular subtypes (luminal A, luminal B, HER-2, and basal) in situ and invasive components. All correlations were statistically significant ($P \leq .05$).

whereas most luminal B carcinomas were classified as intermediate grade (53%); HER-2–overexpressing and basal-like cases were more often of high grade (86% and 77%).

3.2. Evaluation of the invasive component

For the invasive component (Table 2), the luminal A subtype represented 61% of all the tumors. Luminal B and HER-2–overexpressing invasive tumors corresponded to 16% and 12%, respectively, whereas basal-like tumors comprised 8% of the cases. Only 3% of the invasive carcinomas were null phenotype/unclassified. For the luminal A cancer subtype, 93% and 68% of the cases were ER and PgR positive, respectively, whereas for luminal B, 100% were positive for ER and 42% were positive for PgR. Again, all luminal A tumors were negative for HER-2, as expected, and 45% of luminal B cases were positive for this marker. The invasive carcinomas overexpressing HER-2 and negative for hormone receptors were included in the HER-2–overexpressing cancer subtype. In triple-negative basal-like invasive tumors, as described for the in situ component, P-cad expression was the most prevalent basal marker, with 93% positive cases, whereas only 21% and 36% of the cases were positive for EGFR and CK5, respectively (Table 3).

When we studied the expression of basal markers in cancer subtypes other than the basal-like, we found results similar to the ones described for the in situ component of this breast cancer series. Concerning EGFR, exactly the same frequencies were found: 1% and 0 of the cases expressed this receptor in luminal A and B subtypes, respectively, whereas 9% of HER-2–overexpressing tumors coexpressed these 2 tyrosine kinase receptors. CK5 was expressed by 22% of the HER-2–overexpressing invasive carcinomas, whereas only 2% or none of the tumors classified as luminal A or B, respectively, showed CK5 expression. Again, P-cad expression was highly expressed in HER-2–overexpressing tumors (48%), but it was expressed by only 8% of luminal A and 13% of luminal B IBCs.

For Ki-67, the tumors included in the basal-like and luminal B subtypes had the highest proliferative indexes (29% and 25%, respectively). Regarding the histologic grade (Fig. 1), we found that luminal A invasive tumors were often grade I (55%), whereas luminal B lesions were from intermediate (41%) to high grade (38%); once more, HER-2–overexpressing and basal-like tumors were more regularly classified as grade III (95% and 86%, respectively).

3.3. Combined evaluation of the in situ and invasive counterparts in the same patient

Most cases (93%) maintained the molecular classification when the in situ and invasive components were compared (Fig. 2); there were just 13 cases (7%) in which the 2 areas were classified differently (Table 4). One of the cases was unclassified for the in situ component (negative for all markers), but basal-like in the invasive counterpart in which P-cad expression was seen. Both components were high grade. Another case was classified as an in situ luminal A carcinoma but was unclassified in the invasive component because of the absence of expression of both hormone receptors (Fig. 2D). Interestingly, although both components were intermediate grade, the invasive counterpart had a higher proliferative index. Four cases were classified as luminal B for the in situ component but luminal A for invasive counterpart because of the low proliferative index, with the exception of one case that lacked HER-2 expression also in the invasive area. Interestingly, in this case, the loss of expression was accompanied by a difference in the histologic grade: high grade in the in situ portion and grade II in the invasive counterpart.

Finally, 7 cases that were classified as in situ luminal A carcinomas were classified as luminal B in the invasive portion because of a higher proliferative index. Other than the increase in cell proliferation, no alterations were noticed in histologic grade.

In general terms, we can conclude that there are no important modifications of the breast cancer molecular classification in most cases when the transition from an in situ to an invasive carcinoma occurs. However, when we compared the expression of the different biomarkers individually (Table 3), we could find subtle differences between components, which can add some biologic information to the in situ/invasive transition. In the luminal A cases, in addition to the higher proliferative rate in the invasive component in 7 cases, just 3 cases showed P-cad expression in the invasive component. No alterations in hormone receptors were found between in situ and invasive transition. In luminal B tumors, no alterations were found for

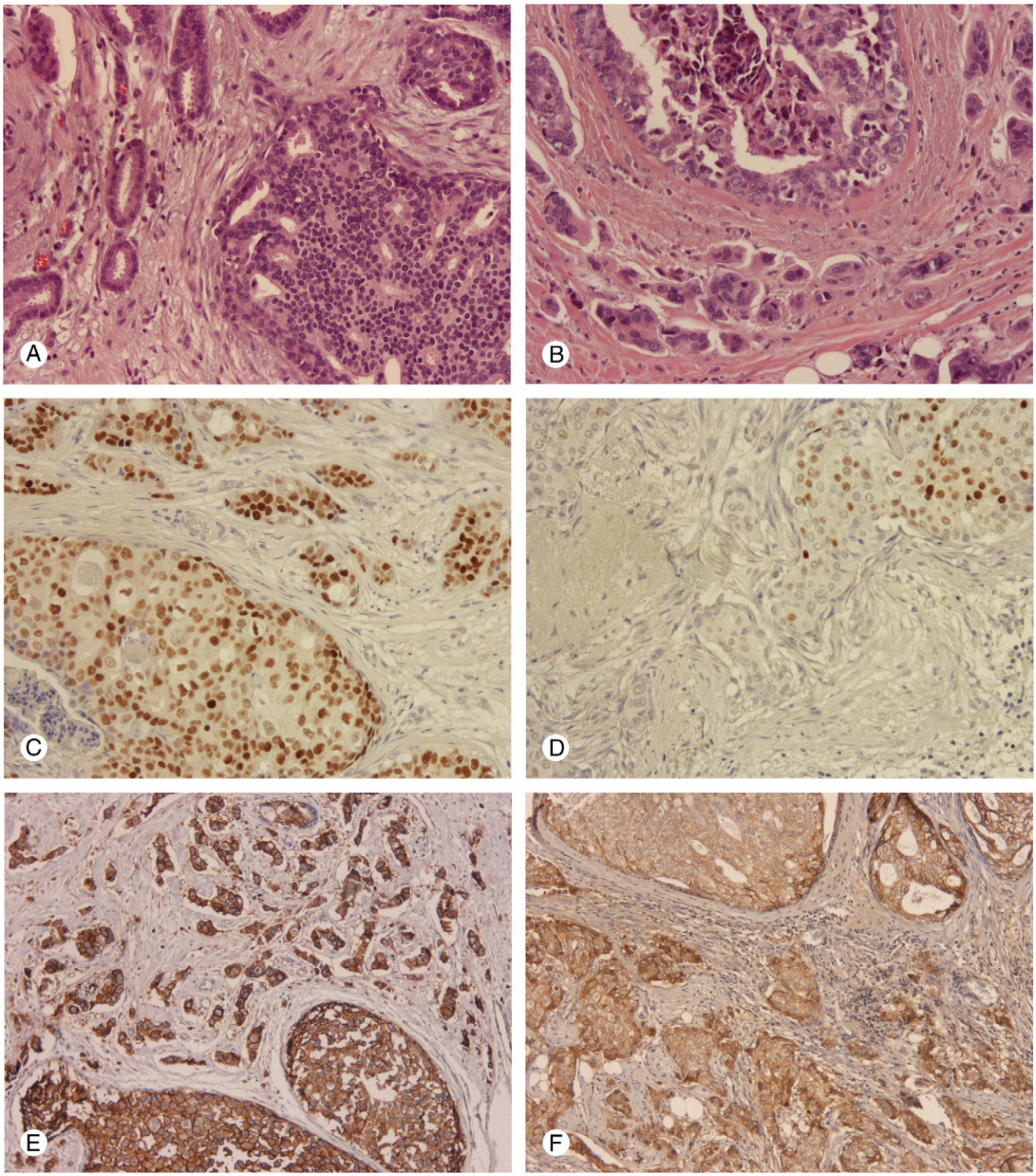

Fig. 2 Expression of proteins studied by IHC staining on TMAs for in situ and invasive components. A and B, H&E staining of low-/I and high-grade/III histologic grade, respectively. C, ER expression. D, Loss of ER expression in invasive component. E, HER-2 staining. F, P-cad staining. Original magnifications: A-D ×200; E, F ×100.

ER expression when the in situ and invasive components were compared. However, there were 4 cases that lost PgR expression in the invasive carcinoma, with the transition from a high-grade in situ carcinoma to a grade II invasive tumor in 2 cases; in the remaining 2, there were no alterations in grade. The other differences were in basal markers, such as CK5 and P-cad, with a loss of 7% and 3% of expression from in situ to invasive tumors, respectively. Regarding the HER-2–overexpression lesions, 7 did not show expression of any basal marker, whereas 14 cases showed concomitant

Table 4 Discordant molecular classifications between in situ and invasive components

No.	In situ component	Invasive component
7	Luminal A	Luminal B
4	Luminal B	Luminal A
1	Luminal A	Unclassified
1	Unclassified	"Basal-like"

expression of EGFR, CK5, or P-cad together with HER-2. Of these, P-cad was the most prevalent. There were 3 cases that gained expression of basal markers in the transition from in situ to invasive carcinoma, namely, 2 with CK5 and 1 with P-cad. Only this last case changed histologic grade (from an intermediate in situ grade to a grade III invasive tumor). In the basal-like subtype, most cases were P-cad positive in both components (11 cases). However, there was 1 case that lost P-cad in the invasive fraction, but because it expressed CK5 in the invasive component, its molecular classification did not change.

Molecular subtypes of in situ and invasive breast cancers did not differ with the histologic grade ($P < .0001$ and $P = .0002$ for in situ and invasive counterparts, respectively). High-grade lesions were associated with the HER-2–overexpressing and basal-like phenotypes in both the in situ and invasive components. Low-grade lesions were frequently of the luminal A phenotype. In the luminal B phenotype, the in situ component was more frequently high grade (53%), whereas the invasive counterpart was intermediate grade (41%).

As mentioned above, the only cases with alterations in molecular classification were not accompanied by differences in histologic grade. However, there were some alterations in histologic grade in individual cases: 9 lesions with intermediate in situ components were grade III in the invasive counterpart, whereas 5 classified as in situ low grade were grade II lesions when we analyzed the invasive component. In cases where there was a decrease in the histologic grade, 12 cases classified as high grade in the in situ component were grade II in the invasive counterpart; 1 case was high grade in the in situ component and grade I in the invasive one, and 10 cases classified as intermediate grade in the in situ component were grade I in the invasive area.

4. Discussion

Two main branches of breast tumorigenesis have been distinguished: one supports the multistep model and the other the theory of parallel disease, where a specific subtype of DCIS matches a specific subtype of invasive breast cancer [12]. In 1997, Grupta et al [25], studying 300 patients with IBC associated with DCIS, demonstrated that the degree of differentiation of DCIS correlated with the grade of the invasive carcinoma and the clinical outcome. They also showed that patients with invasive breast cancer displayed the same mutations as patients with preinvasive and invasive lesions. In fact, recent data [26] demonstrate that the most dramatic alterations in gene expression patterns occur during the transition from normal breast tissue to DCIS [27,28], not from in situ to invasive. In contrast, Tamimi et al [29], studying 272 DCIS and 2249 invasive independent tumors, showed that in situ and invasive phenotypes had different prevalences. These authors found a higher prevalence of luminal B and HER-2–overexpressing profiles among DCIS tumors. However, analyzing independent series of in situ and invasive tumors [17,18], no differences were found in molecular subtype prevalence. So probably, the higher percentage of the HER-2 phenotype in DCIS in the series described by Tamimi et al [29] was attributable to the examination of a mammographically screened population and does not reflect a basis of progression to invasive tumors.

The great advantage of our series of 189 breast carcinomas, which was characterized by several immunohistochemical markers, relies on the existence of in situ and invasive components in the same sample. We classified the in situ and invasive tumors into 4 main molecular subtypes (luminal A, luminal B, HER-2–overexpressing, and basal-like). When the components were compared, we verified that there were no significant differences in the molecular classification of in situ and invasive tumors, which led us to conclude that different molecular subtypes have different progression forms, low-grade in situ tumors evolving into low-grade invasive tumors and high-grade in situ tumors into high-grade invasive tumors [15].

Differences in molecular profiles between the in situ and invasive carcinoma areas were observed in only 13 cases, which seems more likely to be attributable to technical immunohistochemical issues than a reflection of changes in tumor biology. One case classified as luminal A in the in situ component lost ER expression and became unclassified in the invasive component. Another case, which did not express any of the markers used for classification (unclassified) in the in situ component, gained expression of P-cad in the invasive component and could be characterized as having a basal-like phenotype. In the remaining 11 cases, the changes were from luminal A to B or vice versa, and these alterations can be attributed to the fact that the criteria for classification of the luminal B subtype are not well established. Although some luminal B tumors can be identified by their expression of HER-2, the chief biologic distinction between luminal A and B is the proliferative signature, including genes such as Ki-67. Chang and collaborators [23], using 14% as a cutoff, supported Ki-67 as a well-established cell proliferation marker in cancer and emphasized its role as a biomarker candidate for identification of luminal B tumors. We also used this cutoff, which allowed us to distinguish some luminal B tumors that the standard biomarker panel (ER, PgR, and HER-2) did not identify. Interestingly, and although the percentages are close to that of the luminal B subtype, the basal-like tumors had higher proliferative

indices than the other subtypes in both the in situ and invasive components (28% and 29%, respectively). Among these fractions, the invasive one had a higher proliferative rate, which can be associated with an increase in cell proliferation when invasion occurs, and with the poor prognosis associated with this molecular subtype.

An association between histologic grade and molecular phenotype has been demonstrated, with low-grade invasive tumors usually having the luminal A phenotype, whereas high-grade tumors are more prevalent among HER-2–overexpressing and basal-like subtypes [29,30]. Moreover, the HER-2–overexpressing and basal-like subtypes are associated with a poor prognosis. In our series, in in situ breast cancers, HER-2 and basal-like subtypes were more frequently high grade than low or intermediate grade (86% and 77%, respectively). These results were consistent for invasive tumors, because 95% of HER-2+ and 86% of basal-like tumors had high histologic grades. It was interesting to note the percentages of the luminal B type among DCIS and invasive components, where intermediate/II and high grade prevail in both, with 36% and 54% for the in situ component, as well as 41% and 38% for the invasive counterpart, respectively. This similarity between intermediate/grade II and high grade probably is secondary to the cutoff used, which enriched our series in luminal B cases.

We also looked for cases that showed alterations simultaneously in biomarker expression and histologic grade and found 8 cases. It is important to say that these alterations were not accompanied by alterations in the molecular classification. Two cases graded as having an intermediate in situ component were grade III in the invasive component, accompanied by gain of P-cad in one case and its loss in the other. Other 2 cases, with the concordant loss of PgR expression, classified as high grade in the in situ component, were grade II in the invasive counterpart. One case also lost PgR expression but changed from intermediate in situ to grade I in the invasive counterpart. It is also interesting that we had 2 cases that lost PgR expression and 1 basal marker, P-cad or CK5, simultaneously and were classified as high-grade in situ and grade II in the invasive component. Finally, the remaining case lost CK5 and changed from intermediate to grade I.

We have shown that the prevalence of molecularly defined phenotypes did not differ significantly between DCIS and invasive breast cancers; probably, the molecular alterations that drive invasion occur before the morphologic modification of the lesion [31,32]. Dalgin et al [15] also confirmed that the cancer phenotype develops early (in the early hyperplasia or DCIS stage), and each subtype progresses along its own specific pathway, as if each was a distinct disease.

In conclusion, with this work, we showed that it is possible to identify different immunohistochemical profiles of in situ and invasive breast cancer using a small panel of biomarkers (ER, PgR, HER-2, EGFR, CK5, P-cad, and Ki-67) and that the technique of TMA is useful, efficient, and reliable in the characterization and subclassification of a large number of cases. Concerning the comparison of in situ and invasive components, we found that in 176 (93%) of the 189 cases, the molecular classification was identical in the 2 components, which supports the theory of parallel disease; that is, that in the progression of most breast cancer cases, there is a commitment of the in situ subtype carcinoma to a specific subtype of invasive carcinoma. Otherwise, the finding supports the view that the molecular phenotype is established at the DCIS stage. Although there has been an improvement in understanding the pathways of breast tumorigenesis, little is known about the mechanisms associated with the transition from in situ to invasive carcinomas. More than just genetic alterations in the tumor cells, the codependency of epithelial cells and stroma can regulate tumor progression. Recently, it was demonstrated that myoepithelial cells can have a particular role in tumor invasion. Studying normal myoepithelial cells and the ones associated with DCIS, Schnitt [32,33] demonstrated that the last ones differ substantially from the normal, showing down-regulation of genes involved in the normal function of cells and up-regulation of genes associated with invasion. There is an immediate need to characterize new molecules that not only uncover the molecular biology of in situ carcinoma and its transition to invasive breast cancer, but also the transcriptional program that drives the invasive growth of each molecular subtype.

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Publications

Paper II

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Loss of caveolin-1 and gain of MCT4 expression in the tumor stroma

Key events in the progression from an in situ to an invasive breast carcinoma

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Keywords: DCIS, IDC, stroma, tumor progression, breast cancer, Caveolin-1, MCT4, immunohistochemistry

Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; Cav-1, caveolin-1; MCT4, monocarboxylate transporter 4; TMA, tissue microarray; H&E, hematoxylin-eosin; ER, estrogen receptor, PR, progesterone receptor; EGFR, epidermal growth factor receptor; CK, cytokeratin; P-cad, p-cadherin; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization

The progression from in situ to invasive breast carcinoma is still an event poorly understood. However, it has been suggested that interactions between the neoplastic cells and the tumor microenvironment may play an important role in this process. Thus, the determination of differential tumor-stromal metabolic interactions could be an important step in invasiveness.

The expression of stromal Caveolin-1 (Cav-1) has already been implicated in the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC). Additionally, stromal Cav-1 expression has been associated with the expression of stromal monocarboxylate transporter 4 (MCT4) in invasive breast cancer. However, the role of stromal MCT4 in invasiveness has never been explored, neither the association between Cav-1 and MCT4 in the transition from breast DCIS to IDC.

Therefore, our aim was to investigate in a series of breast cancer samples including matched in situ and invasive components, if there was a relationship between stromal Cav-1 and MCT4 in the progression from in situ to invasive carcinoma. We found loss of stromal Cav-1 in the progression to IDC in 75% of the cases. In contrast, MCT4 stromal expression was acquired in 87% of the IDCs. Interestingly, a concomitant loss of Cav-1 and gain of MCT4 was observed in the stroma of 75% of the cases, when matched in situ and invasive carcinomas were compared. These results suggest that alterations in Cav-1 and MCT4 may thus mark a critical point in the progression from in situ to invasive breast cancer.

Introduction

Breast cancer is a heterogeneous and complex disease, encompassing a variety of pathological entities with distinct clinical behaviors. The development of new technologies has offered the opportunity to explore the molecular complexity of human breast carcinomas.[1] However, despite these advances, the mechanisms controlling the transition from an in situ to an invasive carcinoma still remain unclear. Therefore, there is a significant interest in identifying molecular events driving invasive progression, not only to determine at which point the lesion is most likely to progress to malignancy, but also to identify new molecular targets that could trigger the progression at early stages.[1] Several studies have evaluated the gene expression profiles of both ductal carcinomas in situ (DCIS) and invasive ductal carcinomas (IDC),[2-8] but only few compared the in situ and invasive components within the same breast tumor.[5-8] In fact, although some genes have been described as differentially expressed between in situ and invasive components, the majority of the studies failed to demonstrate significant differences between the expression of the codified proteins in the neoplastic epithelial cells of DCIS and IDC.[5,9] Recently, our group, using patient-matched DCIS/IDC tumor samples, showed concordance between in situ and invasive molecular profiles in 94% of the cases.[10] These results suggested that the alterations in

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the tumor microenvironment would have a more important role in the progression from an in situ to an invasive phenotype than the biology of the tumor cells per se, which showed a tendency to be maintained between these both components.

Actually, it is widely accepted that any cancer is a complex system composed not only by neoplastic cells but also by a fine-tuned microenvironment. The first reference to the importance of the microenvironment in cancer comes from Paget, with his proposal of the "seed and soil" hypothesis. Unexpectedly, this concept was "forgotten" and only "recovered" several years later. In breast cancer, tumor microenvironment plays a key role in defining tumor behavior and patient outcome.[11] Gene expression changes that occur in cancer-associated stroma are known to be implicated in prognosis, as well as in cancer progression.[12-14] Ma and colleagues, using gene expression profiling, provided strong evidence that the stroma co-evolves with the epithelial compartments during cancer progression.[12] Analyzing 14 patients with matched normal epithelium, normal stroma, tumor epithelium, and tumor-associated stroma, the authors proposed that microenvironment participates in tumorigenesis even before tumor cells invade the stroma, and it may play an important role in the transition from pre-invasive to invasive growth.[12]

Caveolin-1 (Cav-1), a scaffolding protein mainly involved in vesicular transport, cholesterol homeostasis, and signal transduction, has been associated to the progression from in situ to invasive carcinoma.[15,16] Lisanti and colleagues showed that Cav-1 loss in tumor stroma was associated with an increased risk for early recurrence, metastasis, and decreased overall survival in breast cancer, being also a strong prognostic factor for basal-like breast carcinomas.[17,18] In DCIS, a loss of stromal Cav-1 was predictive of disease recurrence and progression to invasive cancer, since all the patients with loss of Cav-1 recurred, and 80% of them progressed to invasive disease.[16] Moreover, loss of stromal Cav-1 has been related with stromal MCT4 expression in triple-negative breast cancers, also predicting for poor clinical outcome.[19] This protein is a major transporter directly responsible for L-lactate efflux from glycolytic cells and a functional marker of oxidative stress and hypoxia.[20] In addition, it seems to have a role in stromal breast cancer metabolism, since it has been demonstrated that breast cancer cells induce MCT4 overexpression in stromal fibroblasts.[21]

Since stromal expression of MCT4 and the association between Cav-1 and MCT4 had never been implicated in the progression from DCIS to IDC, the aim of this study was to better understand the stromal interactions surrounding in situ and invasive components of breast carcinomas, evaluating the stromal expression of Cav-1 and MCT4 using patient-matched DCIS/IDC tumor samples.

Results

IHC quantification for Cav-1 and MCT4 was performed on each set of the 22 TMA slides using patient-matched DCIS/IDC tumor samples. Data on ER, PgR, HER-2, P-cad, CK5, EGFR, Ki-67 status, histological grade, and lymph node metastases were already available and published for this series.[10]

Cav-1 and MCT4 expression in normal breast

In normal breast, it can be observed that Cav-1 expression was absent from the epithelium, whereas its expression was observed in the stromal component, as previously described.[16-18] MCT4 expression was absent in both epithelial and stromal components, as observed in **Figure 1A**.

Stromal Cav-1 expression in the progression from in situ to invasive carcinoma

In the DCIS component, only 19 cases (13%) showed no Cav-1 expression in the stroma, whereas 55 cases (39%) had moderate expression, and the majority had strong expression of stromal Cav-1 (67 cases, 48%). In the invasive component, the majority (n = 108, 76%) of the cases showed absent Cav-1 expression in the stroma, with only 27 cases (19%) with moderate expression and 7 cases (5%) with strong expression. **Figure 2** represents the expression levels of stromal Cav-1 in in situ and invasive components, where a significant decrease of Cav-1 from DCIS to IDC can be observed. An IHC example of Cav-1in in situ and invasive components is shown in **Figure 3**.

Regarding the progression from in situ to invasive carcinoma, analyzing each case for both matched components, 106 cases (75%) showed loss of stromal Cav-1 expression, whereas 35 (25%) cases maintained protein expression. None of the cases showed gain of stromal Cav-1 expression.

Stromal MCT4 expression in the progression from in situ to invasive carcinoma

Considering the DCIS component, the majority of the cases were negative (n = 131, 93%) (**Fig. 1B**), 10 cases (7%) showed moderate expression, and 5 cases (3%) were classified as strong for stromal MCT4. In the invasive component, a strong expression of MCT4 in the stroma of the majority of the cases (n = 73, 50%) was observed, whereas moderate expression was observed in 63 (43%) cases; in the remaining 11 cases (7%), no expression of stromal MCT4 was observed.

Figure 4 depicts the expression levels of stromal MCT4 in situ and invasive components, showing an increased expression of stromal MCT4 in the invasive component. **Figure 5** represents by IHC the strong MCT4 stromal expression in invasive component.

Concerning the transition from in situ to invasive carcinoma in terms of gains and losses of MCT4 in the stroma, we found that 126 cases (87%) gained expression in the invasive component, 19 cases (13%) maintained, and none lose the expression.

Combining stromal Cav-1/MCT4 in the progression from in situ to invasive carcinoma

Analyzing matched in situ and invasive components for stromal expression of Cav-1 and MCT4 (**Table 1**), it was possible to observe a statistically significant association between the loss of stromal Cav-1 and the concomitant gain of MCT4 in the same case ($P < 0.0001$). Interestingly, 75% of the cases that lost Cav-1 stromal expression in the transition from in situ to invasive cancer also gained MCT4 expression in the stroma. There were only 4 cases (3%) with loss of Cav-1 in the stroma that maintained MCT4 expression and 16 cases (12.5%) that gained MCT4 and maintained Cav-1 stromal expression. In 12 cases (10%), there was the maintenance of stromal expression for both

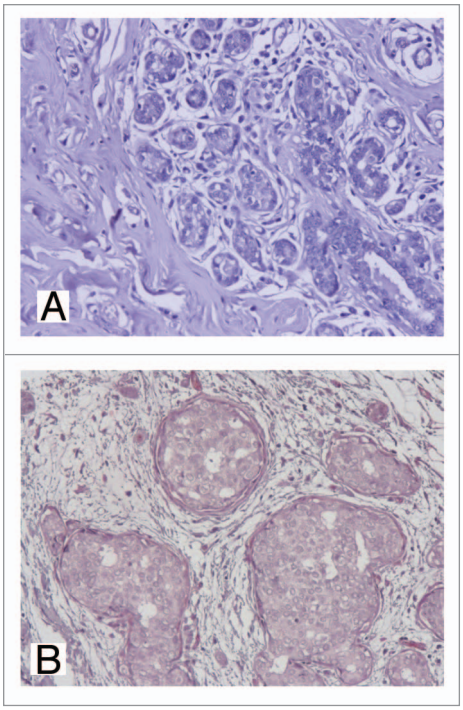

Figure 1. IHC expression of stromal MCT4 in normal and in situ component. Absent stromal MCT4 expression can be observed in normal breast (**A**) and in in situ component (**B**), 200×.

markers. **Figure 6** represents an IHC array with the expression levels of these proteins in the progression from in situ to invasive carcinoma.

Discussion

The mechanisms that mediate the progression from DCIS to IDC in the breast are still largely unknown. However, it is now widely acknowledged that accumulation of genetic anomalies contributes to the acquisition of an increasingly aggressive, invasive, or therapy-resistant tumor phenotype.[1] Nevertheless this knowledge did not improve the predictive power of standard pathological parameters for breast cancer, nor did it explain the mechanisms of invasiveness.

Cav-1 plays an important role in tumor stroma, and recent studies demonstrate that the loss of stromal Cav-1 is associated with advanced tumor and nodal stage, lymphovascular invasion, metastasis, early recurrence, tamoxifen resistance, and reduced progression-free survival in invasive breast cancer.[23-25] Additionally, loss of stromal Cav-1 also has prognostic value in a particularly aggressive subgroup of breast cancers, namely the triple-negative and basal-like breast carcinomas, whereas high levels of this protein were correlated with reduced tumor size, low grade, reduced metastasis, and improved survival.[18,25,26]

Interestingly, loss of stromal Cav-1 also predicts for recurrence and early disease progression in DCIS patients. Witkiewicz et al. reported that 80% of the DCIS patients, which underwent surgical excision and recurred with invasive breast cancers, showed reduced or absent levels of stromal Cav-1 in these tumors.[16] In

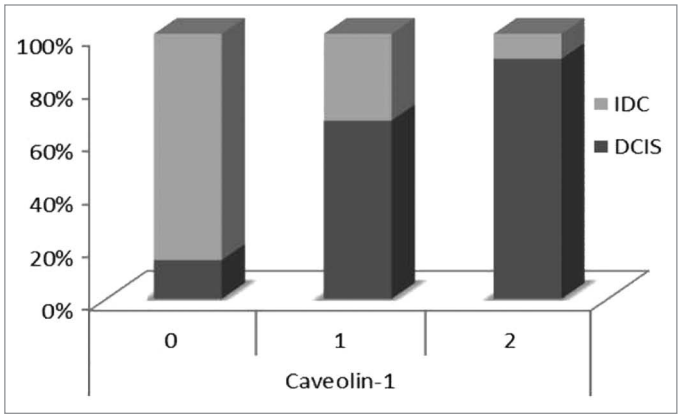

Figure 2. Expression levels of stromal Cav-1 in in situ and invasive components of breast carcinomas. It is possible to notice a significant decrease of Cav-1 stromal expression from DCIS to IDC.

our series, using patient-matched DCIS/IDC tumor samples, it was observed that the majority of the cases showed strong expression of Cav-1 expression in the stroma of DCIS, whereas 76% of the cases showed absent expression for this marker in the stroma of the invasive counterpart. Thus, regarding the progression to invasiveness, it seems that the loss of Cav-1 expression in the stroma is important for tumor invasion.

Actually, it has been already described that loss of Cav-1 in stromal cells may also increase angiogenesis and tumor growth.[15] Goetz et al. demonstrated that in vivo and in vitro expression of Cav-1 in cancer-associated fibroblasts facilitates tumor cells invasion and accelerates the in vitro proliferation and in vivo tumorigenesis.[27,28]

Recent data reveals that loss of Cav-1 induces a metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells—the "reverse Warburg effect", where cancer cells induce upregulation of multiple glycolytic enzymes in neighboring stromal fibroblasts.[23,29,30] Cav-1 is degraded resulting in a loss of stromal Cav-1 expression.[19] At the same time, the breast cancer cells induce MCT4 overexpression in stromal fibroblasts.[19]

MCT4 is a monocarboxylate transporter that functions as a shuttle to extrude L-lactate from cells using aerobic glycolysis for energy metabolism.[20] Although the transporter role of MCT4 has been widely accepted in cancer epithelium, the prognostic value of MCT4 expression is highly compartment-specific and restricted to the tumor stroma, high stromal MCT4 levels being associated to poor patient overall survival.[21,31,32] In our series, analyzing DCIS and IDC separately, an increase of MCT4 expression was observed, since in DCIS the majority of the cases were negative, whereas, in the invasive counterpart, 50% of the cases showed strong expression for MCT4. Considering the progression from in situ to invasive breast carcinoma, using matched DCIS/IDC tumor samples, 87% cases gained MCT4 expression, whereas none showed loss of expression, suggesting that the gain of stroma MCT4 provides evidence for the existence of a stromal–epithelial lactate shuttle which fuels the tumor growth.[21]

Table 1. Association between stromal Cav-1 and MCT4 expression levels in the transition from in situ to invasive breast carcinoma

		MCT4 (in situ to invasive)		
		Loss of expression N (%)	Maintenance of expression N (%)	Gain of expression N (%)
Cav-1 (in situ to invasive)	Loss of expression N (%)	0 (0%)	4 (3%)	94 (75%)
	Maintenance of expression N (%)	0 (0%)	12 (10%)	16 (12.5%)
	Gain of expression N (%)	0 (0%)	0 (0%)	0 (0%)

P value ≤ 0.001

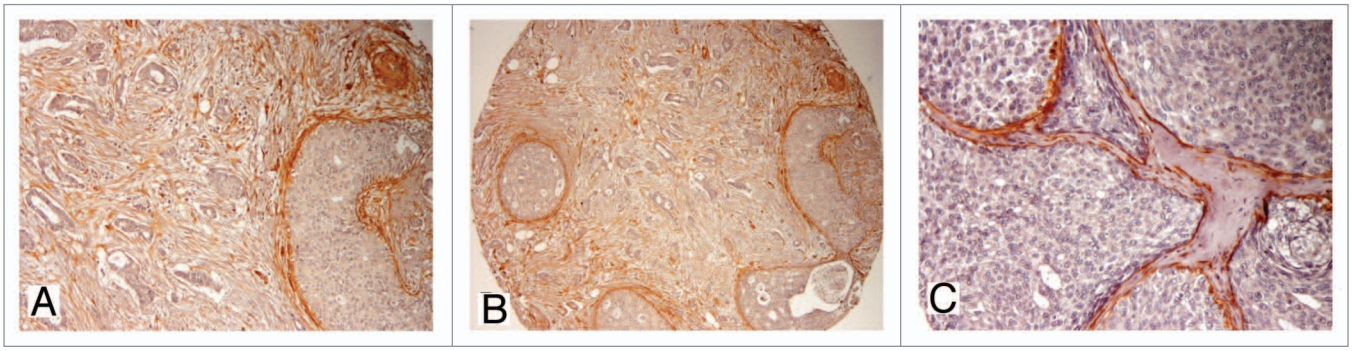

Figure 3. IHC expression of stromal Cav-1 in in situ and invasive components. Note the strong expression of Cav-1 in DCIS, from low (**A and B**, 100× and 200×, respectively) to higher magnification (**C**, 400×).

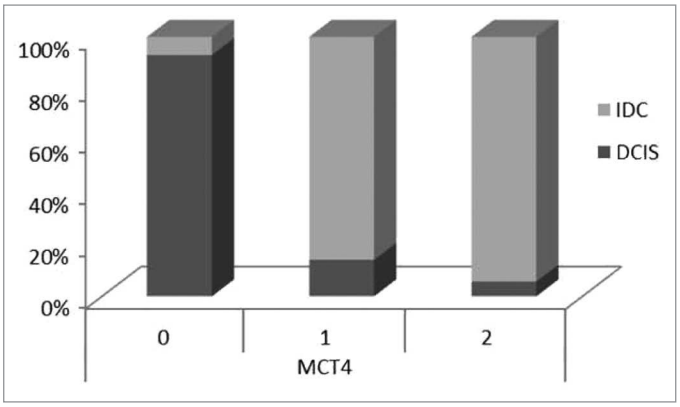

Figure 4. Expression levels of stromal MCT4 in in situ and invasive carcinomas. There is a significant increased expression of stromal MCT4 in the invasive component of breast carcinomas, when compared with DCIS.\

Regarding the relation between MCT4 and Cav-1 expression, Witkiewicz et al.,[19] using 164 invasive breast cancer samples, verified that stromal MCT4 and stromal Cav-1 levels were inversely related, high levels of stromal MCT4 being directly correlated with a loss of stromal Cav-1 immunostaining.[19] Most notably, cases with absent stromal Cav-1 are most likely to present strong stromal staining for MCT4, and, in contrast, cases with strong expression for Cav-1 are most likely to be stromal MCT4 absent.

Nevertheless, studies regarding the role of Cav-1 and MCT4 in the transition from in situ to invasive breast carcinoma were still lacking. In our series, using matched DCIS/IDC and analyzing the concomitant expression of stromal Cav-1 and MCT4, 75% of the cases showed loss of Cav-1 with simultaneous gain of MCT4 in the stroma, suggesting that these events are important for tumor cells to progress and invade.

Our results are explained by the recent "two-compartment tumor metabolism" model and the "reverse Warburg effect", suggesting that the loss of Cav-1 causes the metabolic reprogramming of stromal cells to support the growth of adjacent epithelial tumor cells.[23] In **Figure** 7, a hypothetical model summarizing he alterations in Cav-1 and MCT4 in the stroma of matched in situ and invasive breast carcinoma is shown.

The oxidative stress promoted by the tumor cells induces autophagy in cancer-associated fibroblasts (CAFS) that degrade Cav-1 in the in situ stromal compartment and also secrete energy-rich metabolites, such as L-lactate, ketone bodies, and pyruvate as a consequence of metabolic alterations. During the progression to invasive carcinoma, the loss of Cav-1 induces MCT4 expression due to the amount of energy metabolites, used to promote cancer cell glycolysis, aggressive tumor growth, and, ultimately, invasion of breast cancer cells.

Many of the cited studies quantify one or both markers in breast cancer stroma. However, one potential limitation of the quantification methodologies used is the lack of a clear and reproducible definition of stroma, especially regarding DCIS cases. In our case, since all IHC scoring was performed by the same experienced pathologist, we consider this does not affect internal validity and therefore does not affect the results obtained and conclusions drawn.

In summary, it was shown that the loss of stromal Cav-1 and the concomitant gain of stromal MCT4 have a putative role in

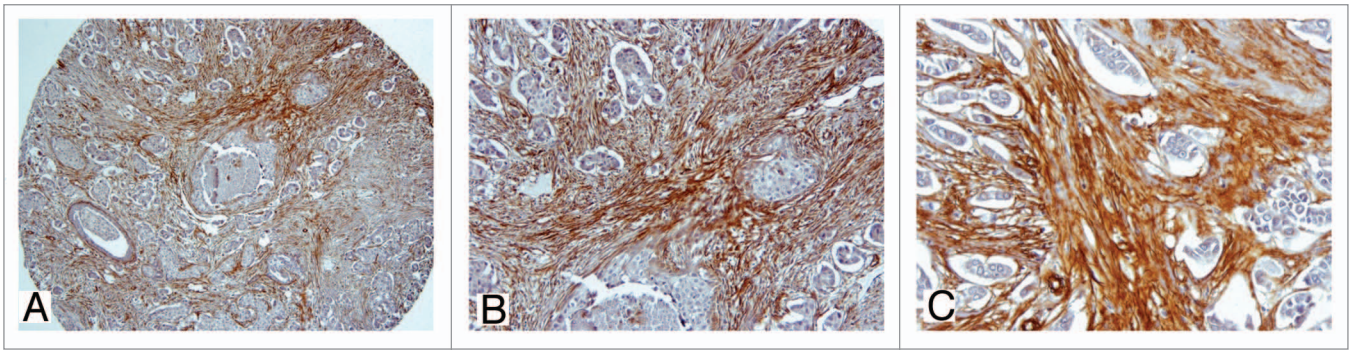

Figure 5. IHC expression of stromal MCT4 in in situ and invasive components. Note the strong MCT4 stromal expression in invasive component, from low (**A and B**, 100× and 200×, respectively) to high magnification (**C**, 400×).

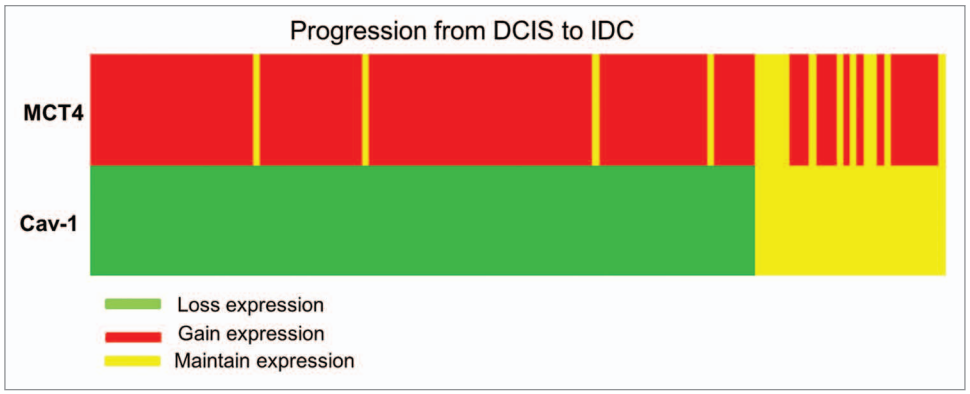

Figure 6. Immunohistochemistry array showing protein expression levels of stromal Cav-1 and MCT4 in the progression from in situ to invasive carcinomas. Cases are arranged along the *x*-axis and proteins are arranged along the *y*-axis. Within the heat map, red represents gain of expression, green represents loss of expression, and yellow represents maintained expression from in situ to invasive carcinoma within the same case.

the transition from in situ to invasive carcinoma of the breast. Therefore, we propose that Cav-1 and MCT4 may represent valuable biomarkers for breast cancer progression. Thus, determining the nature of the cooperation between tumor cells and the microenvironment that leads to invasion could identify therapeutic strategies to prevent the transition from in situ to invasive breast carcinoma.

Material and Methods

Case selection and TMA (tissue microarray) construction

Formalin-fixed and paraffin-embedded samples from 189 tumors, harboring in situ and invasive carcinoma areas in the same block, were consecutively retrieved from our archives. Available data included patient's age and clinicopathological features, such as tumor size and lymph nodes status. Representative areas of the in situ and invasive breast carcinomas were selected on H&E-stained sections and marked on the correspondent individual paraffin block. Two tissue cores (2 mm in diameter) were obtained from each specimen for TMA construction with each TMA block (donor block) and deposited into a paraffin block (receptor block) using a TMA workstation (TMA builder ab1802, Abcam). In each TMA block, non-neoplastic breast and liver tissue cores were also included as controls and TMA guide, respectively. An H&E-stained section from each TMA block was reviewed to confirm the presence of morphological representative areas of the original lesions.

All morphological and IHC assessments were conducted by a pathologist (FS). The study was conducted under the national regulative law for the handling of biological specimens from tumor banks, the samples being exclusively available for research purposes in retrospective studies.

Cav-1 and MCT4 immunohistochemistry

IHC was performed using the HRP labeled polymer (DakoCytomation) for Cav-1 and with the Ultravision Detection System Anti-polyvalent HRP (Lab Vision Corporation) for MCT4. Antigen unmasking was performed using a dilution of 1:100 from a commercially available solution of citrate buffer, pH = 6.0 (Vector Laboratories) at 98 °C. After the antigen retrieval procedure, the slides were washed in a phosphate buffer solution (PBS) and submitted to blockage of the endogenous peroxidase activity by incubation of the slides in a 3% hydrogen peroxide (Panreac) in methanol (Sigma-Aldrich). The slides were further incubated with the primary antibodies for Cav-1 (2297; BD Biosciences, diluted 1:50) and for MCT4 (H-90; Santa Cruz Biotechnology, diluted 1:500), as previously described.[24] All

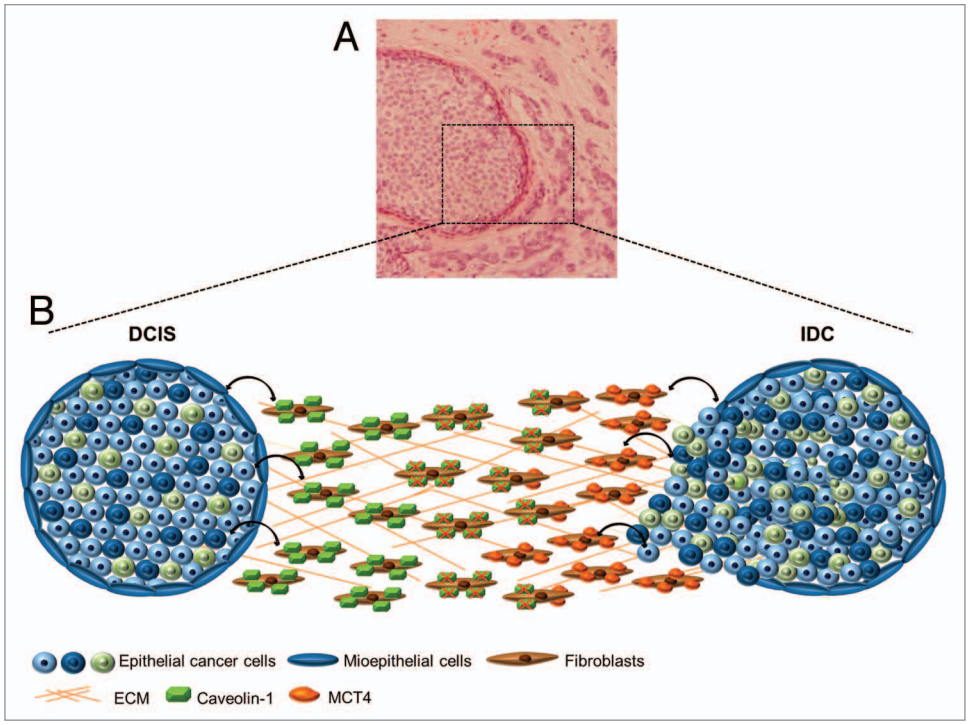

Figure 7. Alterations in Cav-1 and MCT4in the stroma of matched in situ and invasive breast carcinoma. (**A**) H&E-stained tissue section of human breast cancer, showing in situ and invasive components of breast carcinoma (100×). (**B**) Hypothetical model summarizing the importance of Cav-1 and MCT4 in the progression from DCIS to IDC. During the progression to invasive carcinoma, Cav-1 is degraded by oxidative stress-induced autophagy in cancer-associated fibroblasts, resulting in a loss of Cav-1. At the same time, the loss of Cav-1 induces a metabolic reprogramming of stromal cells, where cancer cells induce upregulation of MCT4 by stromal fibroblasts, in invasive counterpart.

reactions were revealed with diaminobenzidine (DAB) chromogen (DakoCytomation).

For both IHC assays, positive controls were included in each run, in order to guarantee the reliability of the assays. Non-neoplastic breast tissues, as well as normal breast surrounding the neoplastic cells, were considered internal controls.

Cav-1 and MCT4 immunohistochemistry evaluation

Cav-1 and MCT4 expression in stroma were evaluated using the previously described methodology.[16-19,21] In summary, Cav-1 and MCT4 were semi-quantitatively scored as negative (0, no staining), weak (1, either diffuse weak or strong staining in less than 30% of stromal cells per core), or strong (2, defined as strong staining in 30% or more of the stromal cells).[21]

Statistical analysis

Statistical analyses were conducted using StatView 5.0 software (SAS Institute Inc). The associations between categorical variables were tested for statistical significance using the chi-square test. A two-tailed significance level of 5% was considered as statistically significant ($P < 0.05$).

Disclose of Potential Conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

Martins D was involved in the construction and characterization of the breast cancer series used in the study and performed the majority of the experimental work and drafted the manuscript. Beça FF and Sousa B have made substantial contributions to the analysis and interpretation of the data. Baltazar F performed some of the immunoassays. Schmitt F was the pathologist that evaluated the immunohistochemical reactions. Paredes J and Schmitt F participated in the design of the study and its coordination and helped to draft the manuscript. All authors had final approval of the submitted and published versions.

Financial Disclosure Statement

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Stromal glycolysis and MCT4 are hallmarks of DCIS progression to invasive breast cancer

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PMID:23907124; http://dx.doi.org/10.4161/cc.25794

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http://dx.doi.org/10.4161/cc.26280

Factors implicated in the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) remain unclear. A new study by Martins et al. demonstrates that there is differential expression of stromal caveolin 1 (Cav-1) and monocarboxylate transporter 4 (MCT4) between DCIS and IDC. Loss of Cav-1 and gain of MCT4 in the stroma are markers of oxidative stress and glycolysis, and this phenotype is found during the progression from DCIS to IDC. Also, loss of stromal Cav-1 and increased stromal MCT4 expression is associated with aggressive disease and poor outcomes in DCIS, IDC, prostate cancer, and head and neck cancer. Metabolic modulators and antioxidants can rescue Cav-1 expression and downregulate MCT4 stromal expression. New clinical trials in DCIS and IDC, studying the efficacy of metabolic modulators, need to be initiated.

Ductal carcinoma in situ (DCIS) is a non-invasive breast cancer where carcinoma cells are confined by the basement membrane of the breast duct. The natural history of DCIS ranges from quiescent disease to the development of invasive ductal carcinoma (IDC), which is a life-threatening condition. DCIS is the precursor lesion for IDC, but the step-wise transformation events that drive its progression are unknown. Only 15% of cases progress from DCIS to IDC, so most DCIS lesions will not impact survival.

Our understanding of tumor metabolism has evolved significantly over the past 5 y, and we now recognize that there is significant stromal–epithelial metabolic coupling. Stromal glycolysis and carcinoma cell mitochondrial metabolism via oxidative phosphorylation (OXPHOS) has been termed the reverse Warburg effect, and this occurs frequently in human cancers. The reverse Warburg effect is associated with aggressive disease and poor outcomes in DCIS, IDC, prostate cancer, gastric cancer, head and neck cancer, and melanoma.[1-4]

A recent study by Martins et al. seeks to address the question of step-wise transformation from DCIS to IDC. They compared the stromal metabolism in regions affected by DCIS and IDC from the same subjects.[5] Samples from over 180 subjects, with evidence of DCIS and IDC in the same tissue blocks were stained for caveolin 1 (Cav-1) and monocarboxylate transporter 4 (MCT4). Loss of Cav-1 is a marker of glycolytic metabolism, stabilization of hypoxia-inducible factor 1 α (HIF-1α), lactate generation, high oxidative stress, and impaired mitochondrial oxidative phosphorylation metabolism. In addition, loss of stromal Cav-1 has been associated with poor outcomes in IDC and high risk of progression from DCIS to IDC.[1] Loss of Cav-1 in IDC compared with DCIS was found in 75% of cases.[5] No increase in Cav-1 expression was found in the progression from DCIS to IDC in any of the samples.

Martins et al. also studied MCT4 expression in the tumor stroma. MCT4 is the main exporter of lactate out of cells. Expression of MCT4 is directly regulated by HIF-1α and lactate generation. High stromal MCT4 expression is associated with poor outcomes in triple-negative breast cancer.[2] Stromal MCT4 expression was low in over 90% of DCIS regions, while low MCT4 levels were observed in less than 10% of IDC regions.[5] Importantly, no decrease in MCT4 expression was found in the progression from DCIS to IDC in any of the samples.[5]

Experimentally, direct contact between breast carcinoma cells and stromal cells is sufficient to induce loss of Cav-1 and gain of MCT4 expression in the stroma, which are markers of a catabolic state with increased glycolysis and oxidative stress[6,7] (**Fig. 1**). FDA-approved medications, such as the mitochondrial inhibitor metformin and the antioxidant N-acetyl cysteine, can reverse cancer stroma Cav-1 and MCT4 expression to that of normal stroma.[6,7]

Thus, stromal transformation to glycolytic metabolism is a new hallmark of progression from DCIS to IDC, and it will be important to evaluate inhibitors of stromal transformation in DCIS and IDC-based clinical trials.

In summary, stromal glycolytic changes induce aggressive cancer experimentally, and human studies have revealed an association between stromal glycolysis and poor outcomes in DCIS and IDC.[1,2] Invasion through the basal membrane of carcinoma cells is sufficient to induce stromal metabolic reprogramming.[5] Hence, metabolic reprogramming of the microenvironment is a key step in cancer progression, and we may be able to better predict which patients will develop IDC in the setting of DCIS, by characterizing stromal metabolism rather than by features of carcinoma cells.

Biomarkers and novel therapies targeting stromal metabolism in DCIS are urgently needed. The results of these studies may allow us to improve outcomes for patients with DCIS that will progress to IDC and avoid unnecessary procedures in patients with indolent DCIS.

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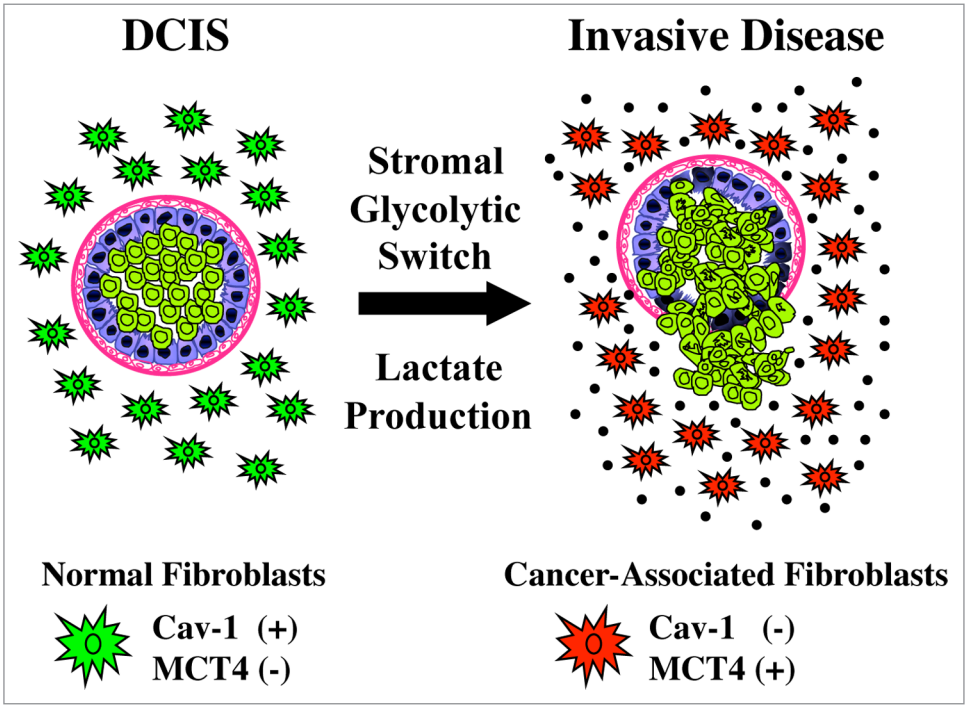

Figure 1. Two-compartment tumor metabolism: Glycolytic stromal cells "fuel" cancer cell aggressiveness during DCIS progression to invasive breast cancer. A catabolic tumor stroma generates and transfers high-energy glycolytic metabolites (monocarboxylates such as L-lactate) to epithelial cancer cells in invasive ductal carcinoma (IDC) with basement membrane invasion. Mitochondrial oxidative phosphorylation metabolism (OXPHOS) is enhanced by this catabolite transfer. Conversely, an intact basement membrane inhibits catabolite transfer from stroma to carcinoma cells in ductal carcinoma in situ (DCIS), and hence OXPHOS metabolism is reduced in carcinoma cells. Caveolin-1 and monocarboxylate transporter 4 (MCT4) expression in the stroma are markers of catabolite transfer to carcinoma cells and OXPHOS metabolism in carcinoma cells. Thus, Cav-1 and MCT4 are biosensors of the glycolytic switch that occurs in the tumor stroma during the transition to malignancy. Normal fibroblasts are oxidative and are Cav-1(+) and MCT4(-). Conversely, cancer-associated fibroblasts are glycolytic and are Cav-1(-) and MCT4(+). Modified, with permission, from reference 8.

Publications

Paper III

P-Cadherin as Prognostic Factor for Loco-Regional Relapse in Breast Cancer

Caderina-P: Valor Prognóstico na Recidiva Loco-regional do Cancro da Mama

Gil FARIA, Maria João CARDOSO, Diana MARTINS, Herberto BETTENCOURT, Isabel AMENDOEIRA, Fernando SCHIMITT

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ABSTRACT

Background: Breast cancer is the most frequent malignant tumor and the leading cause of cancer death in women in Portugal. Due to its relation to an increase in distant metastasis and subsequent death, loco-regional relapse is one major concern in breast cancer women. Several classic prognostic factors as tumour size, nodal stage, histological grade, HER2 status and hormonal receptors have been identified as the most important factors for determining loco-regional relapse, disease free and overall survival. However, there is heterogeneity in prognosis and tumor behaviour in patients with identical disease staging and a similar pattern of expression of known molecular markers, hence the need to discover new prognostic factors. One of the possibilities is P-cadherin, already described by researchers as a possible independent marker of prognosis in breast cancer. The aim of this work was to study in a retrospective series of patients the correlation of P-cadherin expression with loco-regional recurrence in breast cancer women.

Material and methods: We analyzed the clinical records of 1432 consecutive patients with breast cancer and treated in a University Hospital over a 10 year period. Patients with loco-regional relapse (n=101) without prior or simultaneous distant disease were selected as case group. Control group consisted of patients with more than 10 years follow-up and without disease progression. For both groups demographic, clinical, pathological and molecular markers were analyzed. Tissue micro-arrays were constructed to study P-cadherin expression from 86 tumors with available paraffin embedded blocks.

Results: Mean time to recurrence was 41 months and mean survival time after recurrence was 33 months, with a 5-year survival rate of 55%. Tumour size, nodal status and histological grade were identified as independent markers of prognosis. P-cadherin was associated with higher histological grades and hormone negative tumours. P-cadherin was identified as an independent prognostic marker for disease free survival, but not for overall survival.

Conclusion: P-cadherin was related to other known factors of worse prognosis and had an independent relation to disease-free survival. P-cadherin might constitute a novel therapeutic target, but its real biological value is yet to be determined. Doubt persists whether it is an independent marker of tumour behaviour or only a surrogate marker of a set of clinical and molecular features related with worse prognosis.

RESUMO

Introdução: O cancro da mama é o tumor maligno mais frequente e a principal causa de morte nas mulheres em Portugal. Devido à sua relação com a metastização à distância e morte subsequente, a recidiva loco-regional é uma das maiores preocupações no seguimento destas doentes. São conhecidos diversos factores clássicos de prognóstico para recidiva local, tais como o tamanho do tumor, o estádio tumoral, grau histológico, positividade HER2 e a expressão de receptores hormonais. Contudo, existe heterogeneidade no prognóstico e no comportamento do tumor em doentes com estadiamento semelhante e com a mesma expressão de marcadores moleculares de prognóstico. Daí advém a necessidade de descobrir novos factores prognósticos. Uma das possibilidades é a P-caderina, previamente descrita como um possível marcador independente de prognóstico no cancro da mama. O objective deste trabalho foi estudar a correlação da expressão de P-caderina com a recorrência loco-regional do cancro da mama.

Material e métodos: Analisámos os registos clínicos de 1432 doentes consecutivos com cancro da mama e tratados na nossa instituição durante um período de 10 anos. Os doentes com recorrência loco-regional (n=101) sem evidência ou história de metastização à distância foram selecionados como casos. O grupo de controlo consistiu em doentes com mais de 10 anos de seguimento, sem progressão da doença oncológica. Em ambos os grupos foram analisadas variáveis demográficas, clínicas, patológicas e moleculares. Para estudo da expressão da P-caderina, foram construídos Tissue Micro-Arrays a partir de 86 tumores com blocos de parafina disponíveis.

Resultados: O tempo médio livre de doença foi de 41 meses e a sobrevida media após a recorrência foi de 33 meses. A taxa de sobrevivência aos 5 anos foi de 55%. O tamanho do tumor, estadiamento ganglionar e grau histológico foram identificados como marcadores independentes de prognóstico. A P-caderina associou-se com graus histológicos mais altos e tumores sem expressão de receptores hormonais. A P-caderina foi identificada como uma marcador independente de prognóstico para a recidiva livre de doença, mas não para a sobrevivência global.

Conclusão: A P-caderina surgiu associada a outros factores já conhecidos de pior prognóstico e a uma relação independente com a sobrevivência livre de doença. A P-caderina pode vir a constituir um alvo terapêutic a explorar, mas o seu real valor biológico ainda não está determinado. Subsiste a dúvida sobre se a P-caderina é um marcador independente de prognósico ou apenas um marcador de um conjunto de características clínico-patológicas relacionadas com pior prognóstico.

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INTRODUCTION

Breast cancer is the most frequent malignant tumour and the leading cause of cancer related death in woman, with one million cases and half a million deaths each year worldwide.[1] Cumulative individual risk of breast cancer is estimated in 12% (approximately 1 in each 8 women) and the risk of death might be up to 5% (approximately 1 in each 20 women).[2]

Loco-regional relapse of breast cancer is a frequent concern in the treatment of this disease as it has been established as an independent prognostic factor for distant metastasis and subsequent death.[3-6] However, whether it constitutes a cause for distant metastasis or only a marker of an existing risk remains a matter of debate.[5,7,8]

Several clinical and pathological parameters have been used to determine not only prognosis but also the need of adjuvant systemic therapies. The most common of these are: age, size, nodal staging, histological grade, hormonal receptors and HER2 positive.[9-12]

With the development of new microarrays techniques, it became possible to simultaneously analyze thousands of genes and classify tumours according to their profile of genetic expression. As such, a new classification of breast cancer was developed, based on profiles of genetic expression. Five different groups with prognostic differences were identified: luminal A, luminal B, basal, normal-like and HER2.[13]

Although this new classification was based on the hierarchical cluster analysis of genetic expression, some currently available imunohistochemistry markers allow the translation of this classification to the routine pathology practice. Specifically, based on 3 markers (ER, PR and HER2), groups can be divided into luminal type (positive for ER or PR), HER2 positive or triple negative (ER, PR and HER2 negative).[14] The prognostic evaluation of patients in the triple-negative group revealed at least 2 groups of tumors: one of them expressing markers of basal differentiation (CK5, EGFR, P-cadherin) and another without expression of these markers considered unclassified.[13]

Loco-regional relapse is an early and important marker of disease progression. However, regarding patients with identical staging and a similar pattern of expression of molecular markers there is a significant discrepancy in disease progression and prognosis, hence the need to further discover new prognostic factors and stratify risk for disease progression.[12]

One of the molecules used to classify the tumour as basal-like, is P-cadherin, which is associated with increased proliferation and undifferentiated phenotype.[15]

Unlike epithelial cadherin (E-cadherin), P-cadherin expression is usually related to tumorogenic properties, allowing for cellular invasiveness and tumoral aggressive-

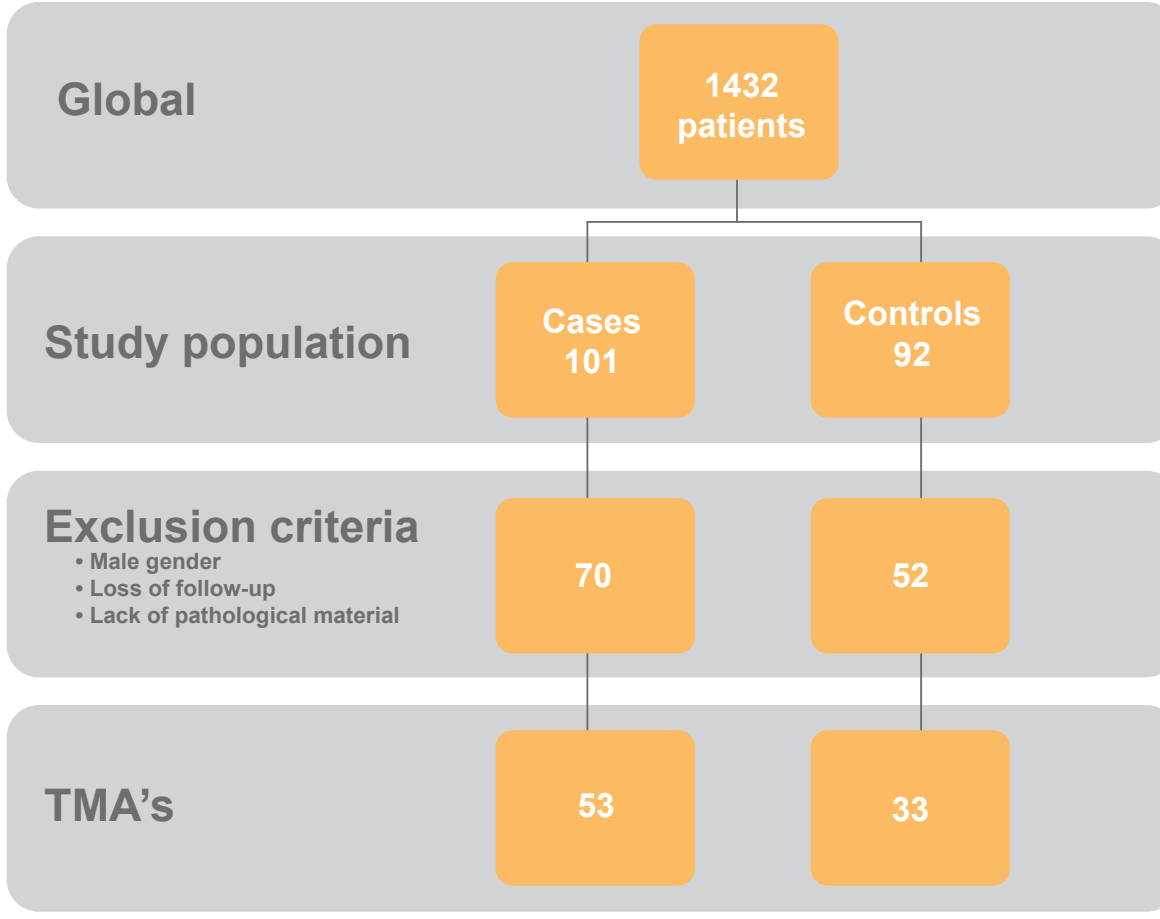

Fig. 1 - Schematic representation of the cohort.

ness, translating into a worst prognosis in breast cancer patients.[16] Its expression is usually associated with other known factors of worse prognosis (high histological grade, high proliferative rate and lack of estrogen receptors).[17,18]

Our objective, in this retrospective series of patients, was to evaluate the correlation of P-cadherin expression in breast cancer loco-regional relapse, disease-free and overall survival.

MATERIAL AND METHODS

We performed a nested case-control study and analyzed the clinical records of 1432 consecutive patients treated and followed at Hospital de São João (University Hospital of Porto Faculty of Medicine) during a 10-year period (January 1st 1997 to December 31st 2006). The case group consisted of all the patients (n=101; 7%) with loco-regional relapse without previous or concurrent systemic progression.

Loco-regional relapse after breast cancer surgery was defined as the onset of histologically confirmed carcinoma at least in one of the following locations: remaining breast tissue; skin, subcutaneous tissue or muscle of ipsilateral thoracic wall; axillary, supraclavicular or internal mammary lymphnodes.[11]

As the majority of loco-regional relapses occurs before 10 years after the initial diagnosis,[19] for control group we selected patients with more than 10 years of follow-up without disease progression: 92 patients surgically treated between January 1st 1997 and June 30th 1998.

Male patients, patients lost to follow-up and those whithout material available for pathological re-evaluation were excluded from the study. Final case group consisted of 70 patients (69.3% of the initial sample) with loco-regional relapse (cases) and 52 patients (56.5% of the initial sample) without disease progression (control group).

Classical clinical and pathological parameters were evaluated in all patients (age, size, type of surgical treatment, TNM staging, histological type, histological grade, presence of associated DCIS, size, lymphatic and venous invasion, Nottingham Prognostic Index [NPI](20) and estrogen receptors. Molecular classification (Luminal, HER2 [HER2(+)/RE(-)/RP(-)], triple negative) and P-cadherin expression were studied using immunohistochemistry in Tissue MicroArrays *(*TMA's*)*.

In our series, only 53 cases and 33 controls had paraffin-embedded blocks available for the construction of TMA's (Fig.1). New sections of the tumour stained with hematoxylin-eosin were undertaken in those blocks. Representative areas were selected and marked for TMA construction.[11]

Representative areas of invasive breast carcinoma were carefully selected on haematoxylin and eosin stained sections and marked on the correspondent individual paraffin blocks. Two tissue cores (2mm in diameter) were obtained from each selected specimen (donor block) and deposited into a paraffin block (receptor block) using a TMA workstation (TMA builder ab1802, Abcam, Cambridge, UK). Twenty-two TMA blocks were constructed, each containing 24 tissue cores (4x6). In each TMA block, non-neoplastic breast and liver tissue cores were also included as controls and TMA guide, respectively. After construction, two 2µm tissue sections were cut and adhered to Superfrost Plus glass slides. An HE stained section from each block was reviewed to confirm the presence of morphological representative areas of the original lesions. Sections were immunostained with primary monoclonal antibodies against ER, PR, HER-2, and P-cad. Immunostaining for ER and HER-2 were performed using the streptavidin-biotin-peroxidase technique (LabVision, Fremont, CA, USA), whereas PR and P-cad immunostaining used the HRP labeled polymer (DakoCytomation, Carpinteria, CA, USA).

Antigen unmasking for ER, PR and HER-2 was carried out using a dilution of 1:100 from a commercially available solution of citrate buffer, pH=6.0 (Vector Laboratories, Burlingame, CA, USA) at 98ºC, whereas a dilution of 1:10 from tris-ethylenediaminetetraacetic (EDTA) solution with pH=9.0 (DakoCytomation) was used for P-cad.

Antigen retrieval time, antibodies, dilutions and suppliers are listed in Table 1. After antigen retrieval procedure, slides were washed in a phosphate buffer solution (PBS), and submitted to blockage of the endogenous peroxidase activity by incubation of the slides in a 3% hydrogen peroxide (Panreac, Spain) in methanol (Sigma-Aldrich). Slides were further incubated with a blocking serum (LabVision Corporation kit) for 15 min and then incubated with the primary antibodies. After washing, slides were incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (LabVision). Diaminobenzidine (DAB) was used as a chromogen (DakoCytomation). For PR and P-cad staining, secondary antibody was associated with HRP labelled polymer (DakoCytomation) and immediately revealed with DAB. Tissues were then counterstaining with Mayer`s haematoxylin, dehydrated and coverslipped

Table 1 - Sources and dilutions of primary antibodies used in this immnohistochemistry

Antibody	Clone	Manufacturer	Incubation time (m)	Dilution	Antigen retrieval (m)
ER	SP1	Neomarkers	30	1:150	30
PR	1A6	Neomarkers	30	1:40	30
HER-2	SP3	Neomarkers	30	1:80	30
P-cad	56	Transduction	60	1:50	30
CK5	XM26	Neomarkers	60	1:50	30

Table 2 - Characterization of the clinical and pathological pattern according to group.

	Cases (n=70)	Controls (n=52)	*p*
Age	N(%)	N(%)	0.84
Age	53.9 (± 16.3)	53,4 (± 11.5)	
Type of Surgery			< 0.001
Lumpectomy	13 (18.6%)	27 (51.9%)	
Mastectomy	57 (81.4%)	25 (48.1%)	OR – 1.69 (1.25 – 2.29)
Surgical Margin			0.751
Distance to margin (mm)	7.95 (± 12.11)	8.69 (± 10.47)	
Chemotherapy			0.059
No	25 (39.1%)	17 (34.0%)	
Adjuvant	22 (34.4%)	27 (54.0%)	
Pre-operative	17 (26.6%)	6 (12.0%)	
Adjuvant Radiotherapy			0.103
No	44 (62.9%)	25 (48.1%)	
Yes	26 (37.1%)	27 (51.9%)	
Hormonal treatment			0.003
No	31 (44.3%)	8 (17.4%)	
Yes	39 (55.7%)	38 (82.6%)	OR = 0.67
TNM Staging			< 0.001
I	6 (8.6%)	23 (45.1%)	
IIa	19 (27.1%)	20 (39.2%)	
IIb	18 (25.7%)	7 (13.7%)	
IIIa	11 (15.7%)	1 (1,9%)	
IIIb	16 (22.9%)	0 (0.0%)	
Histological grade			< 0.001
1	1 (1.5%)	13 (26.5%)	
2	36 (54.5%)	26 (53.1%)	OR = 1.46 (1.16 - 1.83)
3	29 (43.9%)	10 (20.4%)	OR = 2.22 (1.39 – 3.56)
T			< 0.001
1	15 (21.4%)	30 (58.8%)	
2	31 (44.3%)	18 (35.3%)	OR – 1.80 (1.18 – 2.72)
3	8 (11.4%)	3 (5.9%)	OR – 3.82 (1.13 – 12.9)
4	16 (22.8%)	0 (0.0%)	OR – 2.08 (1.43 – 2.97)
N			< 0.001
0	15 (23.9%)	37 (72.5%)	
1	38 (60.3%)	14 (27.5%)	OR = 2.6
2	9 (14.2%)	0 (0.0%)	
3	1 (1.6%)	0 (0.0%)	
Lymphatic invasion			< 0.001
No	21 (33.3%)	38 (77.6%)	
Yes	42 (66.7%)	11 (22.4%)	OR = 2.97
Venous invasion			< 0.001
No	42 (57.7%)	48 (98.0%)	
Yes	20 (32.3%)	1 (2.0%)	OR = 15.8
Nottingham Prognostic Index			< 0.001
Average NPI	5.56 (± 1.42)	3.80 (± 0.98)	
ER			0.004
Neg	25 (42.4%)	6 (15.0%)	
Pos	34 (57.6%)	34 (85.0%)	OR = 0.68
Molecular Classification			0.167
Luminal	46 (70.7%)	41 (85.4%)	
HER2	6 (9.2%)	3 (6.3%)	
Triple Negative	13 (20.0%)	4 (8.3%)	
P-cadherin			0.17
Negative	29 (54.7%)	23 (69.7%)	
Positive	24 (45.3%)	10 (30.3%)	OR = 1.49

Table 3 - Logistic regression for loco-regional relapse

	p	OR
Type of surgery	0.241	0.307
Histological grade	**0.049**	**3.802**
T	**0.034**	**4.672**
N	**0.014**	**8.849**
Lymphatic invasion	0.251	3.215
Venous invasion	0.495	2.857
ER	0.530	0.464

Table 4 - P-cadherin expression according to other markers of prognosis

P-cadherin	Positive (n=34)	Negative (n=52)	*p*
Molecular sub-type			**0.002**
Luminal	19 (55.9%)	46 (88.5%)	
HER2	7 (20.6%)	2 (3.8%)	
Triple negative	8 (23.5%)	4 (7.7%)	
MIB-1			**0.003**
Positive	16 (52.9%)	9 (82.7%)	
Negative	18 (47.1%)	43 (17.3%)	
TNM stage			**0.807**
I	7 (20.6%)	8 (15.4%)	
IIA	11 (36.4%)	15 (28.8%)	
IIB	5 (14.7%)	13 (25.0%)	
IIIA	4 (11.8%)	7 (13.5%)	
IIIB	7 (20.6%)	9 (17.3%)	
Histological grade			**0.008**
1	4 (12.1%)	5 (10.0%)	
2	10 (30.3%)	32 (64.0%)	
3	19 (57.6%)	13 (26.0%)	
T			**0.325**
1	12 (35.3%)	15 (28.8%)	
2	14 (41.2%)	20 (38.5%)	
3	1 (2.9%)	8 (15.4%)	
4	7 (20.6%)	9 (17.3%)	
N			**0.779**
0	13 (39.4%)	17 (36.2%)	
1	16 (48.5%)	25 (53.2%)	
2	4 (12.1%)	4 (8.5%)	
3	0 (0.0%)	1 (2.1%)	

using a permanent mounting solution (Zymed, San Francisco, CA, USA).

Positive controls were included in each run, to guarantee assay reliability. All cases showing an unequivocal nuclear staining for ER and PR in at least 10% of the neoplastic cells were considered positive. We also considered positive cases with membranous staining for P-cad and in at least 10% of the neoplastic cells. HER2 expression was evaluated according to the DakoCytomation HercepTest scoring system. Cases were considered positive (overexpression) for HER2 when immunostaining was classified as 3+. All the samples were blinded and reviewed by the same experienced pathologist.

Statistical analysis was done using SPSS 15.0 (SPSS Inc. Chicago, Illinois, USA). The chi-square contingency test was used for categorical variables and the t-student was used for continuous variables. A p value of less than 0.05 was considered to reflect a significant association. The multivariate analysis was performed with a model of binary logistic regression. The time-dependent variables were analyzed with the Cox regression model and the Kaplan-Meier curves were based on life tables. For the multivariate regression models, we selected the variables with significant association with the outcome on univariate analysis and in the Cox regression model, we also included the type of systemic treatment to check for potential confounding on the effect of P-cadherin.

RESULTS

Mean age at diagnosis was 53.7 years. Mastectomy was the type of surgery performed on the majority of patients (67.2%) and 77% of the patients were classified as stage I or II, according to TNM classification. Predominant histological type was invasive ductal carcinoma (87%) and half the patients (50.8%) had grade II carcinomas (Nottingham grading system). Forty-seven percent of patients had lymphatic invasion and only 19% had venous invasion. Axillary staging was negative (N0) in 45.6%. ER expression was positive in 68.7% of the patients and PR in 52.3% of the patients. The majority of local relapses occurred in the remaining breast tissue or in the thoracic wall (73%) and 56% of the cases were re-excised. After 93 months of mean follow-up, 69% of the patients are alive.

The expression of classical prognostic factors is listed in Table 2. Mastectomy was associated with higher rates of loco-regional relapse but also with the expression of several markers of worse prognosis (larger tumours [70% T1/T2 vs 92.5% for breast conserving surgery; *p*=.02]; nodal metastasis [67.5% vs 27%; *p*<.001], lymphatic [60.5% vs 19.4%; *p*<.001] and venous [24.0% vs 8.3%;*p*=0.05] invasion and higher TNM stages [29.6% stage III/IV vs 10.0%; *p*=.001]).

The specimen's surgical margins were not different between the groups and post-operative radiotherapy was not associated with a decrease in local relapse risk.

Staging was directly associated with relapse risk, as well as histological grade and NPI index. The presence of lymphatic and venous invasion was also strongly associated with loco-regional relapse. Expression of ER was identified as a marker of better prognosis.

Molecular classification was achieved by the use of routine immunohistochemistry and tumours were divided into 3 categories (ER or PR positive, HER2 overexpressing or triple-negative). The majority of patients expressed luminal type markers in both groups (70.7% of the cases vs. 85.4% of the controls). Triple-negative tumours were more frequent in patients with loco-regional relapse (20% vs. 8%) although this value did not reach statistical significance (Table 2).

The logistic regression model (Table 3) identified histological grade, size and nodal invasion as independent markers of prognosis for loco-regional relapse. Once corrected for other prognostic factors, the type of surgery was no longer related with loco-regional relapse.

P-cadherin was positive in 45.3% of cases and 30.3% of controls (*p*=0.17), OR=1.49. There was a positive relation of P-cadherin expression with the non-luminal molecular types and with higher proliferative index (*p*=0.003) as measured by MIB-1. There were no significant relations between P-cadherin expression and other prognostic markers, with the exception of higher histological grade. (Table 4)

P-cadherin expression was related to a significant decrease (*p*=0.017) in disease-free survival, from 90.5 months to 55.2 months (Fig.2). However, these earlier recurrences were not related with a decrease in overall survival (135.5 months vs 136.2 months – Fig.3), despite the differences observed in the 5-year survival rate (82.7% vs 58.3%).

Multivariate analysis of prognostic factors for disease-free survival (Table 5) identified P-cadherin expression as an independent factor of prognosis, (HR=2.1) together with the known classical factors of prognosis: tumor size, nodal staging and histological grade. For overall survival the only identified independent factors were tumor size and histological grade.

DISCUSSION

The research around new molecular markers has rised tremendously not only because they have the capacity to add some information and enhance discriminant power to scores already available[12] with classical markers but also because they can bring some new understanding over the oncological biology or arise as new putative therapeutic targets.[21]

The major limitation of this study is the shortness of the sample, as we could only retrieve 86 tumors for TMA construction. Additionally, this is a retrospective study with a 10-year span and during this period the treatment of breast cancer suffered significant variations.

Table 5 - Cox regression – Overall survival and disease free survival

	Disease-free survival		Overall survival	
	p	HR [95%CI]	p	HR [95%CI]
P-cadherin	**0.047**	**2.108** [1.009; 4.402]	0.129	2.087 [0.807; 5.395]
T	**0.004**	**1.822** [1.217; 2.729]	**0.003**	**2.317** [1.325; 4.053]
N	**< 0.001**	**2.780** [1.609; 4.802]	0.061	1.957 [0.969; 3.954]
Grade	**0.001**	**3.326** [1.666; 6.643]	**< 0.001**	**8.541** [3.188; 22.883]
Molecular class	0.336	0.870 [0.643; 1.178]	0.093	0.688 [0.445; 1.064]
Chemotherapy	0.632	0.891 [0.556; 1.427]	0.270	0.702 [0.374; 1.316]
Hormone therapy	0.234	0.668 [0.344; 1.298]	0.831	0.909 [0.378; 2.188]
Anti-HER2 therapy	0.903	1.057 [0.431; 2.591]	0.980	1.017 [0.275; 3.764]

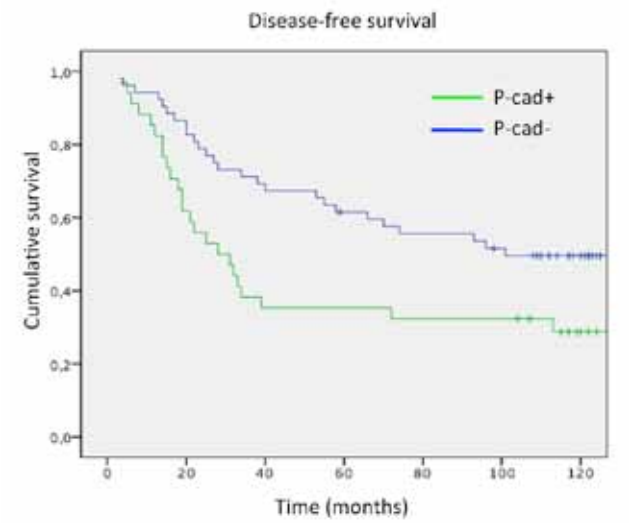

Fig. 2 - Kaplan-Meier curves for disease free survival according to P-cadherin expression.

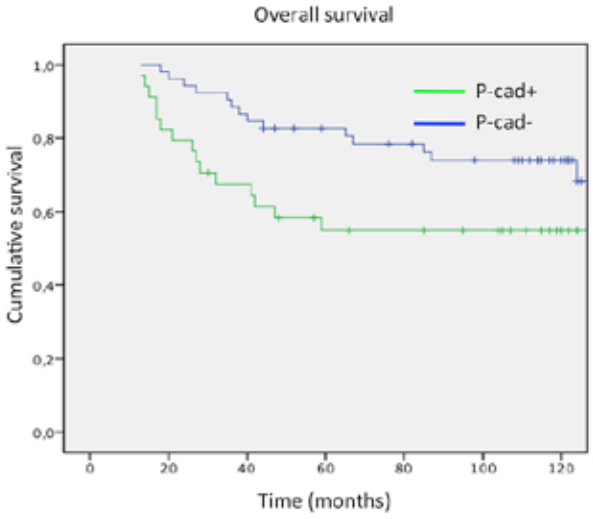

Fig. 3 - Kaplan-Meier curves for overall survival according to P-cadherin expression.

Several studies have reported the risk of local recurrence after breast cancer treatment as being 5-40%.[11,22] Despite therapeutic improvements in the last decade, 40% of the women with local recurrence will have disease progression and eventually death. In our series, local recurrence rate was 7% (101/432) and 54% of these women died from breast cancer, as in most clinical reported studies.[22,23]

Several studies have reported either a similar[24] or increased survival[25] with breast conserving surgery when compared to mastectomy. In our series, breast conserving surgery has a longer median survival (132 vs. 64.5 months for mastectomy). Mastectomy is also related to an increased risk of local relapse (OR = 1.69). However, these results maybe the consequence of a a selection bias, as tumors of patients who had mastectomy, in our series, presented with features of worse prognosis (size, nodal metastasis, histological grade, TNM staging and NPI). Once corrected for these factors, the benefit of conservative surgery is no longer detectable.

Tumor size (p=0.002) and nodal staging (p<0.001) were two important factors of prognosis for local recurrence, which confirms the data of several other studies,[22,26,27] and patients with tumors larger than 5cm had a 4-fold increase in local recurrence as compared with tumors smaller than 2cm (OR=3.82).*4* Also as described in the literature,[28,29] patients with axillary invasion had an almost 3-fold inrease in local recurrence as compared to patients with node-free disease (OR=2.6). According to some authors, axillary invasion might be not just an event related to tumor progression, but a biological marker of tumor aggressiveness[27] independently of tumor size, recurrence type or time-to-recurrence. Also according to several studies,[5,11] there was a significant relation between high histological grade and local recurrence (OR=1.46 for Grade 2 and OR=2.22 for Grade 3; p<0.001). Regarding all well-known factors our results were identical to others of similar series.

In one of the first studies about P-cadherin expression in breast cancer, the molecule was only identified in 4% of invasive breast cancers. In the following studies, its expression was observed in approximately 20% of tumors and inversely related to E-cadherin expression and directly to higher histological grades.[17] With the development of anti-P-cadherin monoclonal antibodies its expression was registered in 30% to 50% of all the invasive ductal cancers.[15,30-32] In our series, P-cadherin was expressed in 39.5% of all cases. P-cadherin was more often positive in patients with local recurrence (OR=1.49; p=0.1), although without a statistically significant difference.

Several studies reported that the P-cadherin expression in cancer cells was directly related to other known factors of worse prognosis, such as: tumor size;[30] histological grade;[17,30,32] ER negativity[17,30,32] and nodal metastization.[30] In multivariate analysis only relation with nodal metastization and histological grade has kept significance.[30] Other reports found no association between P-cadherin expression and tumor size or axillary invasion.[32] These conflicting reports and differing association with known prognostic factors, suggests that P-cadherin might be related to oncological progression of breast cancer, but its real biological behavior is not yet determined.[30] In our series, P-cadherin expression was directly related only with histological grade and ER status.

Several other reports have shown a direct relation of P-cadherin with other known factors of worse prognosis, such as triple-negative type[33-35] and proliferative index.[32,36] In this study, we also confirm these findings of a direct relation between P-cadherin expression and triple-negative tumors (p<0.001) and higher proliferative index (p=0.003), as measured by MIB1.

Several reports observed an inverse relation between P-cadherin expression and hormonal receptors. Most of the P-cadherin expressing tumors lack hormonal receptors expression[17,31,32,37,38] and are positive for HER2, EGFR, higher histological grades and proliferative index, which are asso-

ciated with worse prognosis.[17,30-32] These authors suggest that the hormonal negative state is a requirement to the expression of P-cadherin, probably through the differentiation of luminal type cells into myoepithelial cells where P-cadherin is usually expressed.[32] It has been suggested by some authors that P-cadherin expression in breast cancer cells might represent the differentiation in an embrionary phenotype, similar to the ductal-extremity cells, which are highly proliferative, negative for ER and positive for P-cadherin.[17] Our results, as other before,[30] support this hypothesis as P-cadherin expression was found more often in high histological grade and ER negative cancers.

Although some studies described impairment in survival for patients with P-cadherin expression, in multivariate analysis,[30,31,37] our results only confirm a reduction in disease-free survival (Cox regression; p=0.047), without differences for overall survival (p=0.129). Nevertheless, the Kaplan-Meyer survival curves suggest that there is an effect of P-cadherin on survival, visible at 5-years follow-up and fading progressively, nearly unnoticed at 10 years. Similar data were reported in other studies,[30-32] suggesting this fade-out of effect in long-term follow-up, which explains the lack of association with overall survival but the significant differences of survival at 5 years (82.7% vs 58.3%). More studies directed to the underlying pathophysiology of P-cadherin will be necessary, in order to unravel this effect and to understand the molecular mechanisms and signaling involved in this process.

CONCLUSION

Breast cancer is one of the most prevalent diseases worldwide, being the leading cause of death for cancer in women.[1] In the last few years, the mortality due to breast cancer has been following a downward trend, due to better screening programs and most effective medical care.[39] Local recurrence has been described as a marker of disease progression and an important risk factor for death.[3] As a consequence, several studies have tried to identify risk factors for local recurrence.[11]

One of the most promising markers for loco-regional disease progression seems to be P-cadherin and in the future, it might even constitute a novel therapeutic target.

P-cadherin, in our study was related to other known factors of worse prognosis, was more frequent in non-luminal type tumors and had an independent relation to disease-free survival. Although it did not affect overall survival or relapse rate, it seemed to be associated with earlier relapse and mortality.

The real biological value of P-cadherin is still undetermined raising the question to whether it has an independent relation to tumor behavior or if it constitutes just an indirect marker of a group of clinical and molecular characteristics related to worse prognosis.

CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest.

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Publications

Paper IV

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Oncogenic mutations in gastric cancer with microsatellite instability

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ABSTRACT

Aim: Mitogen-activated protein kinase (*MAPK*) cascade and phosphatidylinositol 3-kinase (*PI3K*) survival pathways are frequently activated in the progression of gastrointestinal malignancies. In this study, we aimed to determine the frequency of gene mutations in members of these pathways – Epithelial Growth Factor Receptor (*EGFR*), *KRAS*, *BRAF*, *PIK3CA* and *MLK3* in a series of 63 gastric carcinomas with high levels of microsatellite instability (MSI).

Methods: Gene mutation analysis was performed by PCR amplification followed by direct sequencing. In selected tumour cases, *EGFR* expression was evaluated by immunohistochemistry. Association studies between molecular data and clinicopathologic characteristics were performed.

Results: Mutations in *EGFR* (3′-untranslated region [UTR] polyA repeat), *KRAS*, *PIK3CA* and *MLK3* genes occurred in 30 (47.6%), 11 (17.5%), 9 (14.3%) and 2 (3.2%) of the MSI gastric cancer (GC) cases, respectively. No *BRAF* or *EGFR* hotspot mutations were identified. Overall, mutations in at least one of these genes were found in 55.6% (35/63) of gastric carcinomas. From those mutant cases 40.0% (14/35) of them had concomitant gene mutations, always involving *EGFR* polyA deletions. Interestingly, we observed significant associations between oncogenic mutations and female gender ($p = 0.046$) old age of diagnosis ($p = 0.001$) and intestinal subtype ($p = 0.043$).

Conclusion: Our results show that MSI gastric carcinoma frequently shows activation of *EGFR-MAPK* and *PI3K* pathways. Within all alterations found, deletions of the A13 repeats of *EGFR* were common, suggesting this molecular event as an important biomarker for stratification of GC patients for treatment with *EGFR* inhibitors.

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1. Introduction

EGFR is a transmembrane protein that homo- or heterodimerizes with other *EGFR* family members at the cell membrane.[1] Receptor dimerisation causes activation of the intrinsic cytoplasmic kinase domain, resulting in the phosphorylation of several tyrosine residues.[2] The active *EGFR* stimulates the *MAPK* cascade and *PI3K* survival pathways.[1] In some neoplasias, such as in non-small cell lung cancer, it has been demonstrated that patients with tumours harbouring structural alterations on the *EGFR* kinase domain could benefit from the pharmacological treatment with *EGFR* inhibitors.[3] However, it is well known that in lung and also colon cancer, the clinical response to *EGFR* inhibitors depends on the tumour genetic profile. Moreover, it has been clearly demonstrated that patients with metastatic colon cancer harbouring mutations in *EGFR* downstream molecules, namely in *KRAS* or *BRAF* genes, are resistant to *EGFR* inhibitors, specifically to the anti-*EGFR* monoclonal antibody cetuximab.[4–7]

Recently, Yuan[14] found a novel mechanism for *EGFR* activation occurring in colon carcinomas with MSI phenotype; mutations in an A13 repeat located at the 3′ (UTR) of gene. Further, mutations in this region of *EGFR* were found to be associated with *EGFR* overexpression.[14]

In GC, and in particular in the MSI subset, data on *EGFR* alterations as well as mutations on its downstream targets, namely those belonging to the *MAPK* and *PI3K* pathways, are very limited. Some authors reported that *EGFR* is over-expressed in a maximum frequency of 38% of GC[8–10] and very few cases were reported to harbour gene structural alterations like gene amplification or mutations.[11–13]

In GC, the *KRAS* gene mutation frequency varies between 3 to 8% and whenever present, *KRAS* mutations normally cluster in the MSI subset (~30% of MSI cases).[15–19] In contrast, others and we found that *BRAF* mutations rarely occur in this type of epithelial cancer.[15,20–23] We have previously reported mutations in *PIK3CA* gene in MSI GC[18] and recently, our group have also reported mutations in the *MLK3* gene, which is a component of the multiprotein *BRAF/RAF1* complex, in MSI gastric and colorectal tumours.[24,25] *MLK3* oncogenic mutations were found in 21% of the MSI gastrointestinal cases and were described to be functionally relevant.[26]

In the present study, we aimed to: (1) determine the frequency of activating oncogenic gene mutations in the 3′-UTR A13 repeat of *EGFR* in mutation hotspots from *EGFR*, *KRAS*, *BRAF* and *PIK3CA*, as well as in the full coding region of *MLK3*, in a series of 63 MSI GC, and (2) to analyse the pattern of these oncogenic mutations to understand the role played by *EGFR* and its downstream targets, namely those belonging to the *MAPK* and *PI3K* pathways in GC progression. Mutations were screened in all cases and associations between the molecular data and the clinicopathologic features of the patients and tumours were also studied.

2. Patients and methods

2.1. Gastric cancer patients and genomic DNA extraction

To assess MSI frequency, 250 GC patients were analysed.[27–29] In total, we selected a series of 63 MSI GC well characterized in terms of clinicopathologic features and geographic area of origin. Microsatellite analysis was evaluated using five quasi-monomorphic mononucleotide repeats BAT-26, BAT-25, NR-24, NR-21 and NR27 cases were considered MSI whenever two or more markers showed instability on five loci considered.[28] The study population was stratified according to area of residence into Central Italy, representing a GC high-risk area, and Southern Italy, representing a GC low-risk area. Tumour and constitutional DNA were extracted from fresh frozen sample tissues using a standard protocol (Gentra Systems, Minneapolis, USA). Pathological examination allowed the selection of areas of neoplastic cells of more than 80%.

2.2. Somatic mutation analysis of EGFR, KRAS, BRAF, PIK3CA and MLK3 oncogenes

For the *EGFR* gene, direct sequencing of the kinase domain (exons 18, 19, 20 and 21) was performed, using a detailed protocol described by Moutinho and colleagues.[13] Structural alterations on the A13 repeat within the 3′-untranslated region of *EGFR* (3′-UTR polyA repeat) gene were also searched, according with the protocol recently described by Yuan[14] in MSI colon cancer. The 3′-UTR polyA repeat was evaluated in normal, as well as, in GC samples. Mutation analysis of *KRAS* codons 12 and 13 and *BRAF* V600E hotspot mutation were performed by PCR amplification and direct sequencing using the protocol used by Oliveira.[30] To search for somatic alterations of *PIK3CA* gene, exons 9 and 20 were sequenced according to the protocol described in detail by Velho.[18] All exons and intron-exon boundaries of *MLK3* gene were screened for mutations. Primer sequences and PCR conditions adopted were recently described.[26] Except for exon 9, a multiplex PCR approach was used to amplify *MLK3* sequence using the QuantiTect Multiplex PCR Kit (Multiplex PCR, Qiagen, Studio City, CA) and following the manufacturer instructions. Purified PCR products were directly sequenced. All sequence alterations in *EGFR*, *KRAS*, *BRAF*, *PIK3CA and MLK3* genes were validated with a second independent PCR.

2.3. EGFR immunohistochemistry

EGFR immunohistochemistry was evaluated on 3 μm sections from formalin-fixed, paraffin-embedded tissue in only two cases with A13 repeat deletion and in one wild-type sample due to the lack of good quality paraffin material for analysis. Epitope retrieval for *EGFR* was performed by proteolytic enzyme digestion (pepsin A, 4 g/l; Sigma–Aldrich, Germany) at 37 °C. After the antigen retrieval procedure, the slides were washed in a phosphate buffer solution (PBS), and submitted to blockage of the endogenous peroxidase activity by incubation of the slides in a 3% hydrogen peroxide (Panreac, Spain) in methanol (Sigma–Aldrich). The slides were further incubated with a blocking serum (LabVision Corporation kit) for 15 min and then incubated with the primary antibody anti-*EGFR* (Zymed, San Francisco, CA, USA, dilution: 1/100; Clone: 31G7) during 60 min. The secondary antibody was associated with HRP labelled polymer (DakoCytomation) and, after that, the slides were immediately revealed with DAB. Tissues were then counterstaining with Mayer's haematoxylin, dehydrated and coverslipped using a permanent mounting solution

(Zymed, San Francisco, CA, USA). Positive and negative controls were included in order to guarantee the reliability of the assay.

2.4. *Statistical analysis*

Analyses were performed using the Statistical Product and Service Solutions, SPSS 14.0 for Windows, 2006, SPSS Inc., Chicago, IL, USA. Statistical associations between the presence of GC oncogenic mutations and clinicopathologic characteristics was assessed by chi-square test for categorical variables and Student's *t*-test or ANOVA test for continuous variables. A p value lower than 0.05 was considered significant.

3. Results

Thirty-five of 63 (55.6%) MSI GC showed oncogenic mutations in at least one of the screened genes (*EGFR*, *KRAS*, *BRAF*, *PIK3CA* and *MLK3*) (Table 1). Representative images of tumour specific oncogenic mutations are shown in Fig. 1. Table 2, summarizes the association studies performed between the presence of oncogenic mutations (independently of the number and type of genes mutated per case) and the clinicopathologic features of patients and tumours.

3.1. *EGFR screening*

We did not find pathogenic mutations in the hotspot regions of *EGFR* (exons 18, 19, 20 and 21). Heterozygous polymorphic variants of *EGFR* were detected in exon 20 (G>A; rs1050171) and in exon 18 (G>A; rs55959834) in 54 and in 2 samples, respectively. However, deletions at the A13 repeat localized into the 3′ UTR of *EGFR* were found in 30/63 (47.6%) of MSI GC. Within the 30 mutated carcinomas, 15 (50%) showed a mononucleotide A deletion, 12 (40%) had a dinucleotide A deletion and, 3 (10%) a trinucleotide A deletion. All 63 normal

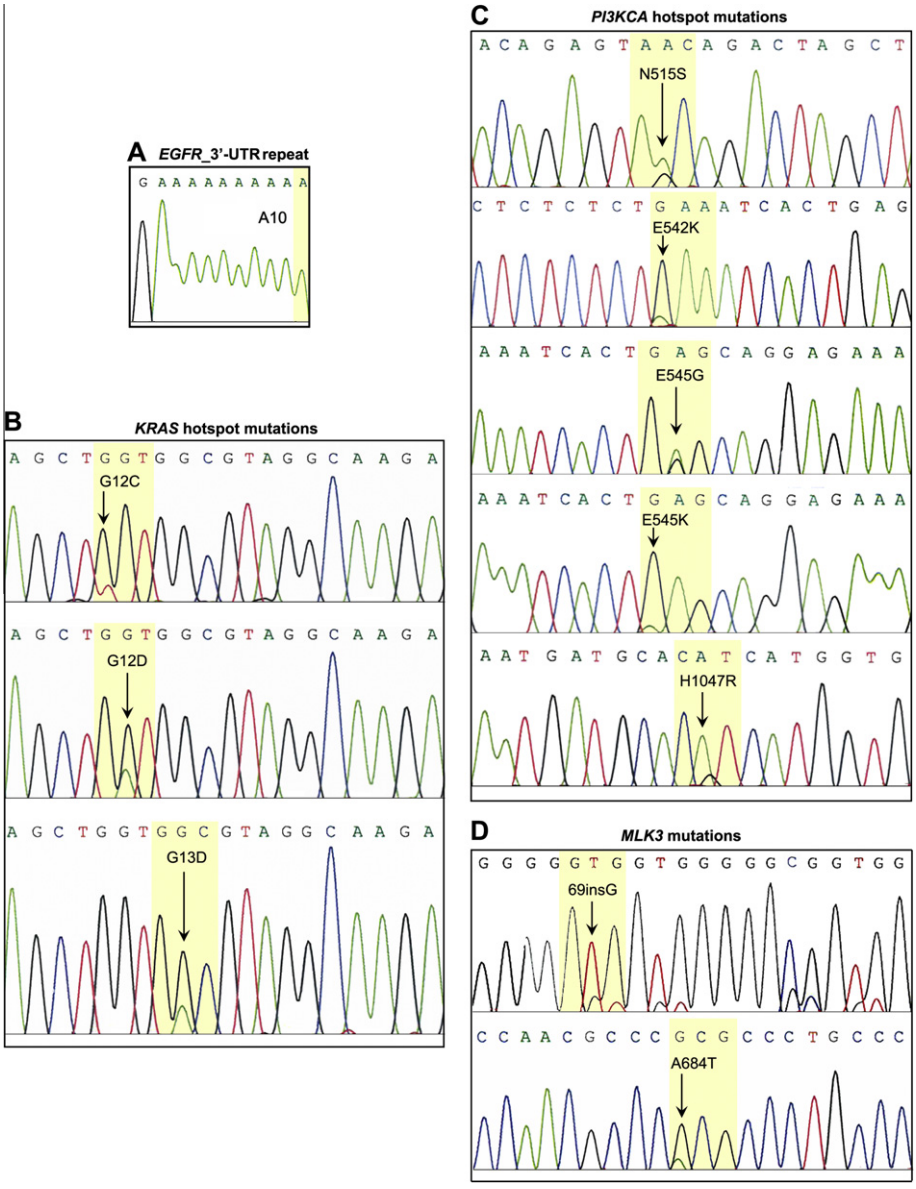

Fig. 1 – Representative images of all oncogenic mutations identified. (A) A10 tumour deletion localized into the 3′ UTR of *EGFR*; (B) *KRAS* hotspot mutations (reverse sequence); (C) *PIK3CA* tumour specific alterations; and (D) *MLK3* gene alterations.

Table 1 – Detailed description of oncogenic mutations identified in the 63 MSI gastric carcinomas.

	Sample code	*MLK3* mutation	*KRAS* mutation	*PIK3CA* mutation	*BRAF* mutation	*EGFR* hotspot mutation	*EGFR* 3′-UTR polyA deletion
1	AU415	wt	wt	**N515S**	wt	wt	$(A)_{13}$ /$(A)_{13}$
2	AB248	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
3	BG191	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{13}$
4	BF070	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{13}$
5	BM175	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
6	BL276	wt	wt	wt	wt	wt	**delA**$(A)_{12}$ /$(A)_{13}$
7	BE163	wt	**G12D**	**H1047R**	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
8	BM213	wt	**G12D**	wt	wt	wt	**delA**$(A)_{13}$/**delA**$(A)_{13}$
9	BC355	wt	**G13D**	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
10	BM406	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
11	BP280	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
12	CD361	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
13	CE120	wt	wt	**H1047R**	wt	wt	**delAAA**$(A)_{10}$/**delAAA**$(A)_{11}$
14	CC442	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
15	CI362	wt	**G12C**	**H1047R**	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
16	CG072	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
17	CI376	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
18	DM272	wt	wt	wt	wt	wt	**delAAA**$(A)_{10}$/**delAAA**$(A)_{10}$
19	DE226	wt	wt	**E545K**	wt	wt	$(A)_{13}$/$(A)_{14}$
20	DM187	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
21	FF336	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{14}$
22	FL141	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
23	FL208	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
24	FF269	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
25	FP285	wt	wt	wt	wt	wt	$(A)_{14}$/$(A)_{14}$
26	FD373	wt	wt	wt	wt	wt	$(A)_{1}$ /$(A)_{14}$
27	GA220	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
28	GE126	wt	**G12D**	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
29	GF364	wt	wt	wt	wt	wt	$(A)_{14}$/$(A)_{14}$
30	JG153	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
31	LG445	wt	**G13D**	wt	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
32	LI266	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
33	MA155	wt	**G12D**	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
34	MG296	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
35	MI052	wt	wt	**H1047R**	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
36	MS399	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
37	MG359	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
38	ME196	wt	wt	wt	wt	wt	**delAA**$(A)_{10}$/**delA**$(A)_{12}$
39	MM110	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
40	MM122	wt	**G12D**	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
41	NV424	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
42	NC410	wt	wt	wt	wt	wt	$(A)_{1}$ /$(A)_{13}$
43	OG249	wt	wt	**E545G**	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
44	PA158	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
45	PB114	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
46	PD192	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{11}$
47	PR209	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
48	PD214	wt	**G12D**	wt	wt	wt	**delA**$(A)_{12}$/**delA**$(A)_{13}$
49	RF421	wt	wt	wt	wt	wt	$(A)_{13}$ /$(A)_{13}$
50	RA125	**A684T**	wt	**E542K**	wt	wt	**delAA**$(A)_{11}$/**delA**$(A)_{12}$
51	RI107	wt	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
52	RA393	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
53	RA270	wt	**G13D**	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
54	RC205	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
55	RL139	wt	wt	**E542K**	wt	wt	$(A)_{13}$/$(A)_{14}$
56	SA13 5	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
57	SE315	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{13}$
58	SA259	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
59	TL130	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
60	TB146	wt	wt	wt	wt	wt	**delA**$(A)_{12}$/$(A)_{13}$
61	VN174	wt	wt	wt	wt	wt	$(A)_{13}$/$(A)_{14}$
62	VP199	**69insG**	wt	wt	wt	wt	**delAA**$(A)_{11}$/**delAA**$(A)_{12}$
63	ZR195	wt	**G13D**	wt	wt	wt	**delAAA**$(A)_{10}$/**delAAA**$(A)_{11}$
Total		2	11	9	0	0	30

Table 2 – Relation between clinicopathologic features of the 63 MSI gastric carcinomas and oncogenic mutations.

	Oncogenic mutation + (35/63; 55.6%)[a]	Oncogenic mutation –(28/63; 44.4%)	P value
Gender			
Male	10 (28.6)	18 (64.3)	0.046
Female	25 (71.4)	10 (35.7)	
Mean age (±SD)	76.7 ± 7.9	67.9 ± 11.4	0.001
Tumor location			
Cardia	4 (11.4)	1 (3.6)	ns
Non-cardia	31 (88.6)	27 (96.4)	
Lauren classification			
Intestinal	31 (88.6)	19 (67.9)	0.043
Non-intestinal	4 (11.4)	9 (32.1)	
Depth of invasion			
pT1–T2	25 (71.4)	14 (50)	ns
pT3–pT4	10 (28.6)	14 (50)	
Lymph node involvement			
pN0–N1	30 (85.7)	20 (71.4)	ns
pN2–pN3	5 (14.3)	8 (28.6)	
Extent of gastrectomy			
Partial	27 (77.1)	20 (71.4)	ns
Total	8 (22.9)	8 (28.6)	
Lymphadenectomy			
D1	15 (42.8)	12 (42.9)	ns
D2/D3	20 (57.2)	16 (57.1)	
Radicality of resection			
R0	32 (91.4)	19 (67.9)	0.017
R1–2	3 (8.6)	9 (32.1)	
Stage grouping			
I–II	23 (65.7)	14 (50)	ns
III–IV	12 (34.3)	14 (50)	
Geographic area			
High incidence	35 (100)	21 (75)	0.001
Low incidence	0 (0)	7 (25)	

Numbers in parentheses are percentage.
[a] Fourteen GC patients carried concomitant somatic mutations.

gastric samples showed a wild-type A13 or A14 repeat of *EGFR* in a homozygous or heterozygous state. According with the findings from the *EGFR* 3′-UTR mutation screening, we verified, by *EGFR* immunohistochemistry, that while two *EGFR* mutant GC (deletion [delAA(A)11]) showed an increased expression of *EGFR* in tumour cells in comparison to the surrounding normal tissue, a wild-type *EGFR* GC case did not display *EGFR* expression in the tumour area (Fig. 2). Due to the lack of good quality paraffin tissue, we can only consider this data as preliminary. This result needs to be validated in a larger series of MSI GC in order to confirm the correlation between the *EGFR* expression and the mutations in A13 deletions in the 3′-UTR polyA repeat of EGFR.

3.2. *KRAS and BRAF mutations*

KRAS mutations were observed in 17.5% (11/63) of MSI GC cases. From these 11 cases harbouring somatic mutations, we found that codon 12 was mutated in 63.6% (7/11) and codon 13 in 36.4% (4/11). All but one *KRAS* mutation localized in codon 12 were G12D. All mutations in codon 13 were G13D. *KRAS* mutations were more frequently found in elderly patients ($p = 0.006$), but no further significant associations were found between other clinicopathologic characteristics and the *KRAS* mutation status (data not show). None of the MSI GC cases under study showed *BRAF* somatic mutations in the hotspot codon previously associated to MSI colorectal carcinomas, the *BRAF* V600E.[31]

3.3. *PIK3CA mutations*

PIK3CA mutations were found in 14.3% (9/63) of the MSI GC studied. Among the mutated cases, eight somatic mutations were located in codons previously described as *PIK3CA* hotspots (codons 542, 545 and 1047). Five *PIK3CA* alterations occurred at the helical domain (codon 515, 542 and 545) and four mutations affected the kinase domain (codon 1047) (Table 1). A novel missense mutation (1544A>G) was identified within the helical domain at codon 515 (N515S). None of these mutations were present in the normal counterpart of these cases.

3.4. *MLK3 mutations*

MLK3 mutations were found in two carcinomas corresponding to a frequency of 3.2% (Table 1): one was a missense mutation (2052G>A) localized in proline-serine-threonine rich domain (A684T) and the other was a frameshift mutation (c.69insG). Besides these two somatic mutations, that were tumour specific, we identified one splice site alteration (c.1069+10C>T) and one missense mutation (2190G>A) (R730H) that were present in both tumour and constitutional DNA, both with unknown pathogenic function. Moreover, we observed two silent *MLK3* sequence variants (225A>G and 2259A>T) that did not change the aminoacid residues of the *MLK3* protein (A75A and P753P, respectively) and were previously described in the normal population.[26]

3.5. *Concomitant oncogenic mutations*

We verified that within the 35 MSI GC harbouring oncogenic mutations, 14 (40.0%) of the mutant cases showed concomitant oncogenic alterations, always involving *EGFR* polyA mutations. From those cases with more than one mutation, seven had *EGFR* A13 repeat deletions and *KRAS* mutations (7/14 – 50%), three showed *EGFR* polyA deletion and a *PIK3CA* mutation (3/14 – 21.4%) and two had *EGFR* deletions and both *KRAS* and *PIK3CA* (2/14 – 14.3%) mutations. In one case we found an *EGFR* deletion and concomitant missense mutations in *MLK3* and *PIK3CA* and in another case an *EGFR* deletion and a *MLK3* mutation (Fig. 3).

4. Discussion

Despite the general advances in diagnosis, standard surgery and chemo- and radio-therapy regimens, the overall outcome of GC patients remains poor, with a 5-year global survival of

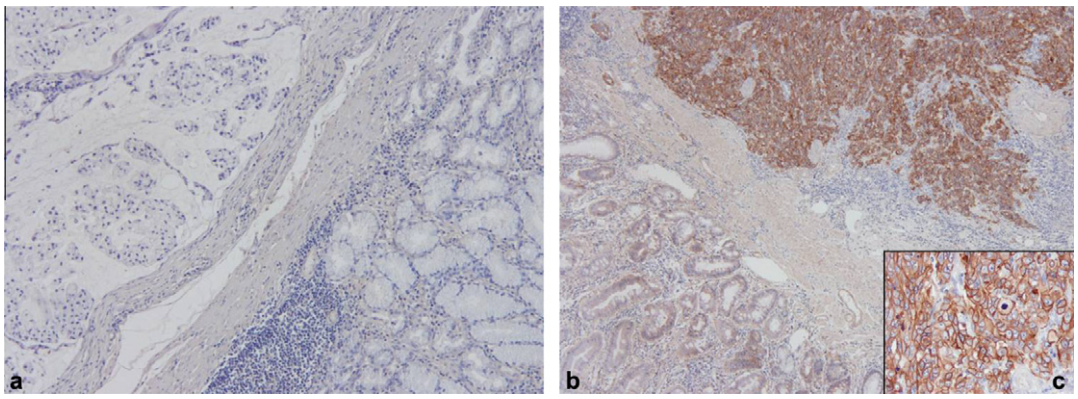

Fig. 2 – *EGFR* immunohistochemistry expression in gastric carcinoma: (a) example of a *EGFR* A13 tract wild-type GC case showing negative *EGFR* expression (amplification 100X); (b) *EGFR* overexpression in tumour area in a GC case displaying *EGFR* A10 deletion in 50× amplification and (c) in 400× amplification.

about 26%.[32] Various multimodal therapy regimens are used to improve the prognosis of GC patients, but no single chemo-therapy regimen is recognized as a global standard.[33] The high prevalence of incurable disease and the poor overall survival of GC patients create the urgent need to find new therapeutic tools for GC treatment.

In patients with advanced GC, several clinical trials were conducted, namely testing *EGFR* inhibitors. However, the overall response to *EGFR* tyrosine kinase inhibitors was poor. Among *EGFR* tyrosine kinase inhibitors, it has been demonstrated that gefinitib treatment showed a particularly low response rate (18%) in advanced GC while erlotinib was completely inactive.[34,35] Recently, more encouraging results were obtained in GC treatment using a combination of multiple chemotherapies.[36–41] As example, patients treated with cetuximab and FUFOX/FOLFOX (5-fluorouracil, oxaliplatin, folinic acid) or FOLFIRI (5-fluorouracil, irinotecan and folinic acid) had a significant higher response rate (62%) when compared with GC patients treated with cetuximab alone (5%).[36–38] Furthermore, when cetuximab was associated with oxaliplatin/leucovorin and 5-fluorouracil, 50% of the patients showed a positive response rate.[39,40] A similar response rate (65%) has been reported in metastatic GC treated with cetuximab plus oxaliplatin/ folinic acid, in which *EGFR* overexpression has been documented.[41] However, until now it was impossible to predict which GC patients will respond to anti-*EGFR* therapy. In other words, no predictive biomarkers are available for clinicians to use in the stratification of GC patients or to predict treatment benefit. Taking into account data from treatment of colon cancer patients, where the response to anti-*EGFR* therapy depends on the genetic make-up of the tumours, namely on the *EGFR* activation status and on the mutation profile of members of *EGFR* and *MAPK* signalling pathways,[42,43] we decided to study these same players in MSI GC.

Different molecular mechanisms underlie *EGFR* protein activation, such as somatic mutations or gene amplification, both leading to an abnormal receptor function. In our series

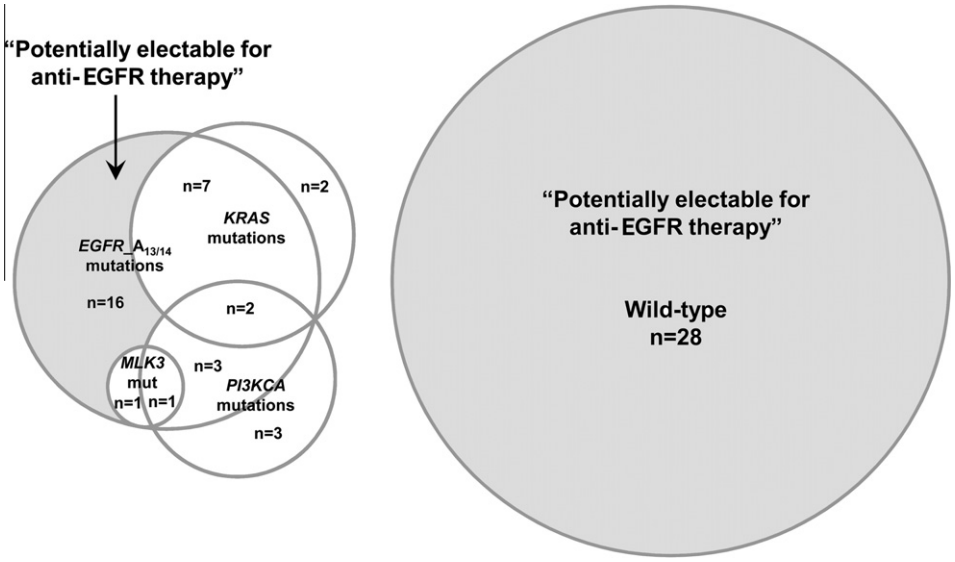

Fig. 3 – Schematic representation of the distribution of the oncogenic mutations identified in the present study (occurring as single events or as concomitant alterations). Oncogenic mutations were found in 35 GC cases but fourteen of them showed more than one mutagenic event in genes belonging to the *EGFR* signalling pathway.

of MSI GC, we did not find hotspot *EGFR* mutations. This finding was not surprising as *EGFR* mutations have been rarely described in GC and gene amplification was only described in a low frequency of cases.[13] Therefore, the presence of other mechanisms directly or indirectly leading to *EGFR* activation were herein investigated.

Since it was recently demonstrated that a polyA tract at the 3′-UTR of *EGFR* was prone to harbour deletions in MSI colon cancer,[14] we searched for this type of alterations in our series of MSI GC. We found alterations at this site in a high frequency (47.6%) of cases. A higher frequency (69%) was found for this type of alteration in MSI colon cancer.[14] Furthermore, and similarly to *EGFR* 3′-UTR polyA tract mutant colon cancer cases, we also verified high level of *EGFR* expression in two tumour samples harbouring this type of alteration. These results suggest *EGFR* A13 repeat mutations as a putative molecular marker to select GC patients for anti-*EGFR* therapies, namely anti-*EGFR* monoclonal antibodies as verified in colon cancer.[14,45]

As previous mentioned, in metastatic colorectal carcinomas, a response to anti-*EGFR* therapies was only observed in patients with tumours without *KRAS* or *BRAF* mutations.[4,6,17,43–45] In particular, the presence of a *KRAS* mutation is well established as predictive of non-response to anti-*EGFR* antibody and shorter patient survival.[5,6] In contrast, the clinical evidence concerning *PIK3CA* mutations is not yet straightforward in this cancer setting.[46,47]

Given the similarities in the mutation spectrum of colorectal carcinomas and MSI gastric carcinomas,[18] we hypothesize that MSI GC cases without alterations in *KRAS* and *BRAF*, and possibly in other members of the *MAPK* (*MLK3*) or *PI3K* (*PIK3CA*) pathways may benefit from this therapeutic approach. Taking in account this hypothesis, we determined the frequency of mutations in all these genes in order to verify their putative importance as biomarkers for therapy assessment.

In the present study, *KRAS* mutations were found in 17.5% of MSI GC cases, while no *BRAF* mutations were detected in these samples, in keeping with data on record.[15,16,18,20–22] These data elicit *KRAS* but not *BRAF* activation as potential biomarkers for therapy assessment in MSI GC. Similarly to *BRAF*, somatic alterations at the *MLK3* gene were very rarely found (3.2%), as previously described in another sample setting of MSI GC.[26] *PIK3CA* mutations were found in a non-neglectable frequency of MSI GC cases (14.3%, respectively) and despite no proof exists showing that mutant *PIK3CA* tumours may not respond to anti-*EGFR* therapy, it is important to characterize its mutation status. The above presented data suggest, in light of the current knowledge on the treatment of gastrointestinal cancer with anti-*EGFR* therapy, that MSI GC patients: (1) can also be stratified according to the molecular profile of their tumours; (2) present, in over 45% of the cases mutations at the *EGFR* 3′-UTR that potentially lead to *EGFR* overexpression which turn these cases in a subset of potentially responsive tumours to anti-*EGFR* treatment; (3) display *KRAS* mutations in nearly 20% of the cases making these GC patients as potentially resistant to anti-*EGFR* therapies; (4) harbour *PI3KCA* mutations in almost 15% of the cases but the use of this information is of limited interest, and finally; (5) lack or display very low frequencies of *BRAF* and *MLK3* mutations and therefore these markers will not improve a future panel of genes to be tested in potentially eligible patients for anti-*EGFR* therapy. Another layer of information that was obtained in our multiple gene mutation screening approach, showed that over one third (40%) of the mutant MSI GC cases accumulate mutations in more than one gene, demonstrating, on one hand, that multiple molecules within or targeted by the *EGFR* pathway are involved in GC progression; but on the other hand, reducing the number of cases potentially benefitting from anti-*EGFR* therapy. In detail, as 30% (9/30) of *EGFR* mutated cases also display *KRAS* mutations, thus it is predictable that 70% (21/30) of mutant *EGFR* patients will benefit from *EGFR* inhibitors (Fig. 3). Furthermore, another set of the patients harbouring *EGFR* mutations also displayed a *PIK3CA* mutation which, although unproved, may also result in resistance to therapy (Fig. 3).

In GC, cases harbouring concomitant oncogenic mutations in the *MAPK* cascade is about 3%.[18] Our data showed that 40% of cases have concomitant oncogenic mutations (Fig. 3). In our series, the higher percentage of cases with multiple oncogenic mutations is related to the additional screening of the *EGFR* polyA tract and *MLK3* gene, never evaluated so far. In fact, considering only *PIK3CA* and *KRAS* alterations, the number of cases with concomitant mutations is much lower and similar to the frequency reported by Velho and colleagues.[18] The accumulation of *EGFR* 3′-UTR and/or *PIK3CA*/*KRAS*/*MLK3* mutations within MSI gastric carcinomas suggest a possible synergistic effect in the signalling pathways associated to the activation of these genes in GC development/ progression.

Our results support the proposal to implement a multigene screening approach to predict *EGFR*-targeted therapy. We show that within MSI GC three groups can be individualized: Group A: cases that are wild-type for all genes under screening (*EGFR* and genes of the *MAPK* and *PI3K* pathways) – 44.4% (28/63); Group B: cases with oncogenic alterations restricted to *EGFR* - 25.4% (16/63); Group C: cases with oncogenic mutations in genes of the *MAPK* and *PI3K* pathways with or without concomitant *EGFR* alterations -30.2% (19/63). Overall, cases from groups A and B (69.8%) are potentially eligible for anti-*EGFR* therapy. Moreover, from the experience with colorectal cancer it is expectable that cases with oncogenic mutations affecting *MAPK* and *PI3K* signalling pathways GC patients will be non-responders for anti-*EGFR* therapies.

The advantage to identify deletion at the *EGFR* A13 repeat region is to select a novel category of patients that potentially benefit from *EGFR* inhibitors as therapeutic approach. Probably other genetic mechanisms, to date unknown, can also activated the *EGFR* pathway even in cases that belong, in our series, to the wild-type *EGFR* group.

We verified that oncogenic mutations mostly occur in MSI GC patient with older age at diagnosis and of the intestinal subtype, supporting that within MSI GC different molecular pathways are activated in order to generate specific phenotypes.

Palli and colleagues showed in another study performed in the same geographical region (Tuscany region) an association between MSI, positive family history of GC and high consumption of red meat and nitrates, suggesting that environmental factors, such as nutritional habits may play a key

role in inducing genomic instability and an increased risk for GC. Our data demonstrated that oncogenic mutations related significantly with this high incidence area and this probably is associated with the presence of MSI phenotype.[27,48]

In conclusion, our results show that alterations at multiple molecules within or targeted by the *EGFR* pathway are frequent in MSI GC and, that within members of the pathway, deletions of the A13 repeat of *EGFR* were the most common genetic event followed by *KRAS* and *PIK3CA* mutations. Furthermore, in over one third of the cases, concomitant mutations occur in distinct genes, always involving *EGFR*. More importantly, our results open new avenues regarding the stratification of MSI GC patients for anti-*EGFR* therapies and pinpoint a non-neglectable group of cases that may benefit from this therapeutic approach.

Conflict of interest statement

None declared.

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Publications

Paper V

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Alterations in Vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions

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Abstract

Background: Breast cancer is a heterogeneous disease associated with different patient prognosis and responses to therapy. Vitamin D has been emerging as a potential treatment for cancer, as it has been demonstrated that it modulates proliferation, apoptosis, invasion and metastasis, among others. It acts mostly through the Vitamin D receptor (VDR) and the synthesis and degradation of this hormone are regulated by the enzymes CYP27B1 and CYP24A1, respectively. We aimed to study the expression of these three proteins by immunohistochemistry in a series of breast lesions.

Methods: We have used a cohort comprising normal breast, benign mammary lesions, carcinomas *in situ* and invasive carcinomas and assessed the expression of the VDR, CYP27B1 and CYP24A1 by immunohistochemistry.

Results: The results that we have obtained show that all proteins are expressed in the various breast tissues, although at different amounts. The VDR was frequently expressed in benign lesions (93.5%) and its levels of expression were diminished in invasive tumours (56.2%). Additionally, the VDR was strongly associated with the oestrogen receptor positivity in breast carcinomas. CYP27B1 expression is slightly lower in invasive carcinomas (44.6%) than in benign lesions (55.8%). In contrast, CYP24A1 expression was augmented in carcinomas (56.0% in *in situ* and 53.7% in invasive carcinomas) when compared with that in benign lesions (19.0%).

Conclusions: From this study, we conclude that there is a deregulation of the Vitamin D signalling and metabolic pathways in breast cancer, favouring tumour progression. Thus, during mammary malignant transformation, tumour cells lose their ability to synthesize the active form of Vitamin D and respond to VDR-mediated Vitamin D effects, while increasing their ability to degrade this hormone.

Background

Breast cancer is one of the major causes of death by cancer in women worldwide [1]. Nowadays, breast cancer is no longer considered to be a single disease, but is rather comprised of distinct tumour subtypes displaying different clinical outcomes [2]. Over the lifetime of the individual, in order to a tumour to develop it needs a combination of low-penetrance genetic factors and environmental aspects. Ultimately, cancer results from alterations in the control of the complex balance of proliferation, differentiation and programmed cell death [3] and these processes appear to be regulated by intrinsic and extrinsic factors, like niche signals, hormonal and dietary aspects, among others [4], [5].

Vitamin D is a lipid soluble substance that belongs to the family of secosteroid hormones. Its physiological role has been classically associated with calcium regulation and phosphate transport in bone metabolism. Apart from this endocrine role, subsequent studies have widened the range of functions for Vitamin D and this

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has been particularly important in the field of cancer research. Several authors have demonstrated, in various models of cancer (including the breast), the ability of Vitamin D to perform autocrine and paracrine functions. Specifically, it has been demonstrated the capacity to modulate cancer features, namely proliferation and differentiation [6], apoptosis [7], angiogenesis [8], invasion and metastasis [9].

Vitamin D exerts most of its biological activities by binding to a specific high-affinity receptor, the Vitamin D Receptor (VDR), that was first identified in a breast cancer cell line in 1979 [10]. The VDR belongs to the superfamily of nuclear receptors for steroid hormones and regulates gene expression by acting as a ligand-activated transcription factor [11]. Several studies have demonstrated that the VDR knockout mice display a higher incidence rate of carcinogen-induced preneoplastic breast lesions when compared with their littermates [12], [13]. These reports highlight the importance of the VDR deficiency in sensitizing the mammary gland to transformation in response to a carcinogenic agent. Immunohistochemical studies have confirmed that the VDR is expressed in samples from normal breast tissues [14] and also in breast cancer biopsy specimens [15]. Because the VDR is expressed in the mammary gland and Vitamin D has been shown to display anticarcinogenic properties, this hormone has emerged as a promising targeted therapy. But in order to keep the homeostasis of the organism the amount of circulating Vitamin D has to be tightly regulated. This is a very complex process, in which the main components are the enzymes 1α-hydroxylase/CYP27B1 (encoded by the gene *CYP27B1*) and 24-hydroxylase/CYP24A1 (encoded by the gene *CYP24A1*). CYP27B1 is responsible for the synthesis of the biologically active form of Vitamin D (1,25-dihydroxyvitamin D), whereas CYP24A1 mediates the catabolism of Vitamin D [16]. Several studies have focused their attention in the comparison of the levels of these enzymes in normal and tumour tissue. It has been observed that both CYP27B1 and CYP24A1 are up-regulated in breast tumours when compared with normal tissue. However, deregulated expression of CYP24A1 seems to abrogate the effects of CYP27B1, resulting in the degradation of Vitamin D to less active metabolites [17]. In contrast, a recent paper has demonstrated that CYP27B1 mRNA in breast tumours is decreased in comparison with normal mammary tissue [18]. Despite these findings, no reports regarding the expression by immunohistochemistry of the VDR, CYP27B1 and CYP24A1 in the mammary gland have been described. The main purpose of this work was to perform an immunohistochemical study of the expression of the VDR, CYP27B1 and CYP24A1 in a comprehensive series of human breast tissues comprised of normal breast, benign mammary lesions, carcinomas *in situ* and invasive breast carcinomas.

Methods

Patient's selection and Tissue Microarray construction

We have studied a cohort of 379 benign lesion samples and 189 cases of carcinomas *in situ*, collected from the archives of the Pathology Department of General Hospital of UNIMED in Araçatuba, Brazil. Three hundred and fifty cases of invasive breast carcinomas were retrieved from the archives of the Pathology Department of the Federal University of Santa Catarina, Florianópolis, Brazil (161 cases) and from the Pathology Department of General Hospital of UNIMED in Araçatuba, Brazil (189 tumour samples). This last series of 189 invasive carcinomas contains, in the same block, the aforementioned carcinomas *in situ*. Additionally, 29 cases of normal breast tissue were included in the study. The normal breast tissue, carcinomas *in situ* and invasive tumour samples were collected between 1994 and 2004. The series of benign lesions was collected between 2002 and 2006.

Representative areas of the different lesions were carefully selected on the H&E-stained sections, by 2 pathologists (DV and LAV) and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each selected specimen and precisely deposited into a recipient paraffin block using a TMA (Tissue Microarray) workstation (TMA builder, LabVision Corporation, USA). Several TMA blocks were constructed (40 for the invasive breast carcinomas, 22 for the carcinomas *in situ* and 17 for the benign lesions), each containing 24 tissue cores, arranged in a 4×6 sector. In each TMA block, at least 3 nonneoplastic breast tissue cores were also included as controls and 1 core of a non-breast sample (we have used testicular and liver tissues). To homogenize the paraffin of the receptor block and the paraffin of the cores extracted from the donor blocks, the TMAs were kept at 37°C for 3 hours. After construction, 2-μm tissue sections were cut and adhered to Superfrost Plus glass slides. An H&E-stained section from each block was reviewed to confirm the presence of morphological representative areas of the original lesions.

The present study has been conducted under the national regulative law for the usage of biological specimens from tumour banks, where the samples are exclusively available for research purposes in the case of retrospective studies.

Immunohistochemistry

Immunohistochemical staining for Oestrogen Receptor (ER), HER2 and CK5 (Cytokeratin 5) was performed

using the streptavidin-biotin-peroxidase technique (LabVision Corporation) in each set of glass slides comprising the TMAs, whereas P-cadherin (P-cad), EGFR (Epidermal Growth Factor Receptor) and Progesterone Receptor (PgR) used the HRP labelled polymer (Dako-Cytomation, USA) as described elsewhere [19]. Antigen unmasking for VDR was performed using a solution of pepsin A (4 g/L; Sigma-Aldrich) for 30 minutes at 37°C. Epitope retrieval for CYP27B1 and CYP24A1 was performed using a dilution of 1:100 of citrate buffer, pH = 6.0 (Vector Laboratories, Burlingame, CA, USA) at 98°C for 30 minutes. The antigen retrieval times, antibodies, dilutions and suppliers are listed in Table 1. Primary antibody incubation was performed overnight at 4°C for VDR and CYP24A1 and for 1 h at room temperature for CYP27B1. After washes, the slides were incubated with secondary antibody associated with HRP labelled polymer (ImmunoLogic, The Netherlands) for VDR or incubated with biotinylated secondary antibody (Santa Cruz, USA) followed by streptavidin-conjugated peroxidase (Labvision) during 15 min for CYP24A1 and CYP27B1, and immediately revealed with DAB (Dako-Cytomation). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and cover-slipped using a permanent mounting solution (Zymed, USA). Positive and negative controls were included in each run in order to guarantee the reliability of the assays. Paraffin sections of a basal cell carcinoma of the skin, normal colon and normal liver were used as positive controls for VDR, CYP27B1 and CYP24A1 expression, respectively.

Scoring and statistical analysis

The evaluation of the immunohistochemical results was performed by three pathologists (FS, FM and LAV). VDR nuclear expression was evaluated using the H-score method: intensity ranked from 1 to 3 (1 - weak, 2 - moderate, 3 - strong), and extension ranked from 1 to 10 (1 - 0-10% cells, 2 - 11-20% cells and so on, until a maximum score of 10) [20]. The scores for intensity and extension were multiplied and the following criterion was applied: the cases were considered negative when ranging from 1 to 4; samples ranking from 5 to 30 were considered to be positive. Considering the lack of previous reports for the immunohistochemical evaluation of the CYP27B1 and CYP24A1, we considered the cases to be positive only when cytoplasmic staining was observed. The other markers were scored as described in previous studies from our group [19], [21].

The Statview 5.0 software package (SAS Institute, USA) was used for all statistical analysis. Correlations between discrete variables were performed using the chi-square test and analysis of variance was employed to search for associations between continuous and discrete variables. In all analyses, a p value < 0.05 was considered to be statistically significant.

Cell culture and Western blotting

MDA-MB-231 breast cancer cells were grown in complete Dulbecco's Modified Eagle Medium (DMEM) in the presence of 10% foetal bovine serum (Invitrogen, USA). Treatments with Vitamin D 100 nM (Cayman Chemical, USA) and ethanol (vehicle) were performed for 72 h, while the treatment with PTH (Parathyroid Hormone) (Sigma-Aldrich, Germany) 100 nM and water (vehicle) were performed for 4 h. Total cell lysates were obtained and the samples were separated in an SDS-polyacrylamide gel. After blotting into a nitrocellulose membrane (GE Healthcare Life Sciences, UK), staining for CYP27B1 and CYP24A1 was performed using the antibodies (Santa Cruz, USA) presented on Table 1 overnight at a dilution of 1:200. After washes, the membranes were incubated with a mouse anti-goat HRP secondary antibody (Santa Cruz) and were revealed with ECL (GE Healthcare Life Sciences).

RNA extraction and Real-time PCR

RNA was extracted from formalin-fixed paraffin-embedded breast lesions using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, USA), according to the manufacturer's protocol. After extraction, RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, Germany) following the manufacturer's instructions. Finally, real-time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems, USA), using 2 mL of cDNA and in accordance to the manufacturer's protocol. The TaqMan Gene Expression Assays used were Hs00172113_m1 (VDR), Hs00168017_m1 (CYP27B1) and Hs00167999_m1 (CYP24A1). Reactions were performed using standard cycle parameters on an ABI PRISM Sequence 7000 Detection System (Applied

Table 1 Sources and dilutions of primary antibodies related to the Vitamin D metabolism used in this study for immunohistochemistry

Antibody	Clone	Manufacturer	Time of incubation (min)	Dilution	Antigen retrieval (min)
VDR	9A7γE10.4	Calbiochem, Germany	overnight	1:50	30
CYP27B1	C12	Santa Cruz, USA	60	1:200	30
CYP24A1	C18	Santa Cruz, USA	overnight	1:75	30

Biosystems). Relative transcript levels were determined using Human GAPDH Endogenous Control (Applied Biosystems) as an internal reference. Differences between the breast tissue samples were determined using comparative delta C_T method [22]. All reactions were done in triplicate and expressed as mean of the values from three separate experiments.

Results

VDR, CYP27B1 and CYP24A1 immunohistochemical staining

The expression patterns of the VDR, CYP27B1 and CYP24A1 have been evaluated by immunohistochemistry in 947 breast tissue samples arranged in 79 TMAs. From this set of cases, some samples could not be assessed due to the fact that either the core had fallen out or it did not have enough biological material to study. In all TMAs, positive and negative cases were obtained for each protein. The immunostainings for these markers had been previously validated in whole tissue sections with an overall agreement of 90%. A panel with representative immunostainings for each protein in different breast tissues is shown in Figure 1. We have observed that the VDR displays nuclear staining, as would be expected from a nuclear receptor which acts as a transcription factor. Considering CYP27B1 and CYP24A1 expression, nothing has ever been described on their expression status in the mammary gland, as far as we know. This is the first report showing the expression of these two enzymes in breast lesions. These proteins present cytoplasmic and granular staining, which could reflect their mitochondrial localisation. All proteins (VDR, CYP27B1 and CYP24A1) have been found to be expressed in all lesions studied and also in the normal breast tissue, although at different levels.

The differential expression of CYP27B1 and CYP24A1 was technically validated. MDA-MB-231 breast cancer cells have been treated with PTH 100 nM and Vitamin D 100 nM and total cell lysates have been extracted. Western blotting analysis has confirmed the expression of CYP27B1 and CYP24A1 upon treatment with the aforementioned hormones (Additional file 1: Figure S1). Additionally, using a group of randomly selected tissue samples, RNA was isolated and used in real-time PCR to confirm the immunohistochemical results (Additional file 2: Table S1). Our results have shown that positive cases in the TMAs displayed cDNA amplification in the real-time PCR and the opposite situation was observed for cases where no staining was present in the TMAs.

Expression of the VDR, CYP27B1 and CYP24A1 in benign lesions of the mammary gland

In order to study the VDR, CYP27B1 and CYP24A1 expression in benign lesions of the mammary gland, we have evaluated 379 cases arranged in 17 TMAs. The series consisted of a variety of breast lesions, namely usual and atypical ductal hyperplasias (UDH represent 20.1%, corresponding to 76 samples; while ADH represent 5.4%, corresponding to 21 samples), columnar cell lesions (CCL - 25.6% of cases, corresponding to 97 samples), papillomatosis (16.9% of cases, corresponding to 64 samples) and adenosis (17.2% of cases, corresponding to 65 samples). The percentage of immunoreactive cases for the VDR was very high (93.5%, corresponding to 259 cases out of 277). Regarding the expression of CYP27B1, we have observed 55.8% of positive cases, corresponding to 173 lesions out of 310. Concerning CYP24A1 expression, we have detected 62 positive cases out of 327 samples (19.0%). Amongst all lesions, ADH cases were overall less immunoreactive to the three proteins.

We have correlated the histological classification of the benign lesions with the VDR, CYP27B1 and CYP24A1 expression, but no significant associations have been found (see Table 2 for further details).

Expression of the VDR, CYP27B1 and CYP24A1 in breast carcinomas *in situ*

A fully characterized series of 189 breast carcinomas *in situ* arranged in 22 TMAs was assessed for the expression patterns of VDR, CYP27B1 and CYP24A1. For the VDR, we have observed that 62 cases out of 131 cases (47.3%) displayed staining for this protein. Concerning CYP27B1 expression, we have encountered positive staining in 66.4% of the cases (91 out of 137 samples); whereas CYP24A1 expression was observed in 56.0% of the tumours (70 out of 125 cases).

We have also assessed the expression of other breast cancer biomarkers in our cohort (ER, HER2 and PgR and basal markers as defined by our group [19] and others [23]) and looked for the existence of correlations between the expression of the Vitamin D partners and these molecular markers (Table 3). ER expression has been observed in 117 cases (61.9%), HER2 protein was present in 37 cases (15.6%) and PgR expression was detected in 90 cases (47.6%). We have also tested our series for basal markers and have obtained the following results: EGFR expression is present in 10 cases (5.3%), CK5 is positive in 15 cases (7.9%) and P-cadherin was observed in 36 samples (19.0%). Expression of the VDR correlated positively with ER status ($p = 0.0227$), with a higher percentage of VDR-positive cases among the ER-positive tumours - 74.2% (46 out of 62 cases). Additionally, we have seen that there is an inverse correlation between the expression of the VDR and P-cadherin ($p = 0.0078$). CYP27B1 expression only presented an inverse correlation ($p = 0.0295$) with EGFR expression, but the number of cases positive for EGFR was very low. No statistically significant associations have been observed between CYP24A1 expression and the markers studied.

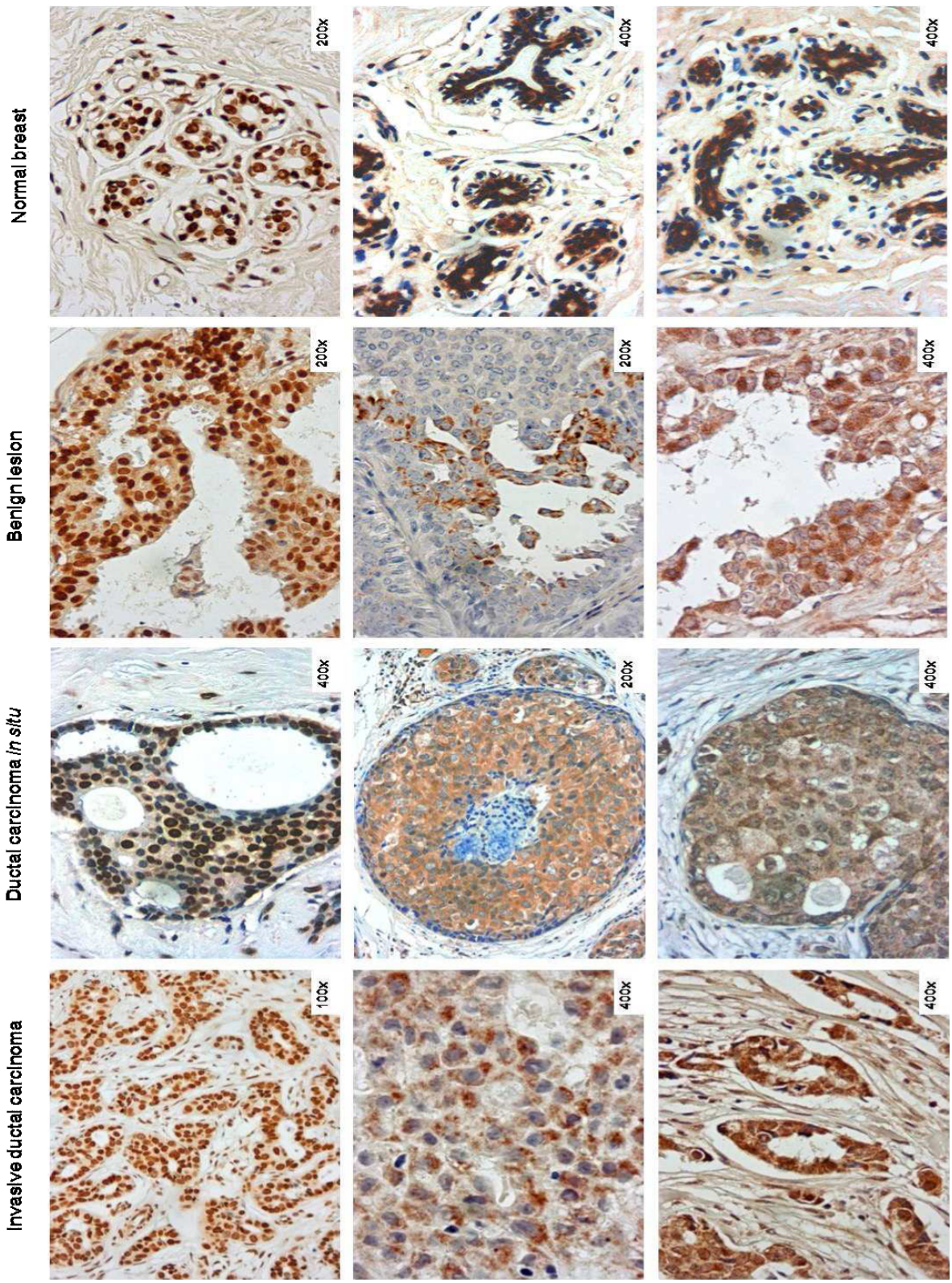

Figure 1 Immunohistochemical staining for the VDR, CYP27B1 and CYP24A1 in the different types of breast tissue

Table 2 VDR, CYP27B1 and CYP24A1 expression in the various types of benign breast lesions

	VDR		CYP27B1		CYP24A1	
	+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
Usual ductal hyperplasia	84 (92.3)	7 (7.7)	57 (55.9)	45 (44.1)	23 (20.5)	89 (79.5)
Atypical ductal hyperplasia	9 (81.8)	2 (18.2)	4 (36.4)	7 (63.6)	1 (7.1)	13 (92.9)
Columnar cell lesions	63 (95.5)	3 (4.5)	43 (55.8)	34 (44.2)	13 (16.5)	66 (83.5)
Papillomatosis	45 (95.7)	2 (4.3)	30 (56.6)	23 (43.4)	9 (17.0)	44 (83.0)
Adenosis	49 (92.5)	4 (7.5)	32 (55.2)	26 (44.8)	13 (22.0)	46 (78)
p value	**0.4847**		**0.7994**		**0.6842**	

Expression of the VDR, CYP27B1 and CYP24A1 in invasive mammary carcinomas

We have evaluated 350 cases of invasive breast carcinomas arranged in 40 TMAs. The cohort corresponds to 189 cases of the series for which there was an *in situ* component in the adjacent area of the invasive tumour and an additional series of 161 cases of invasive breast carcinomas. Positive staining for the VDR has been observed in 56.2% of the cases (172 out of 306 cases). Regarding CYP27B1 expression, 44.6% of cases were positive (123 out of 276 samples), whereas 53.7% of cases (151 out of 281 tumours) presented positivity for CYP24A1.

Next, we searched for associations between the expression of Vitamin D partners and the expression of the molecular markers mentioned in the previous section (Table 4). We have obtained 197 cases (56.3%) positive for ER, 70 cases (20%) for HER2 and 143 cases (40.9%) for PgR. As for basal markers, we have observed that 13 cases (3.7%) were positive for EGFR expression, 48 cases (13.7%) presented positivity for CK5 and 93 cases (26.6%) stained for P-cadherin.

A statistically significant association was observed between the VDR-positive cases and ER-positive cases (p = 0.0002). Additionally, VDR-positive cases have also been significantly correlated with HER2-negative cases (p = 0.0238), but this is probably due to the low number of positive cases for HER2 in our series of mammary carcinomas. CYP27B1 expression presented no significant associations with any of the markers analyzed. PgR was the only marker that displayed an inverse correlation with CYP24A1: specifically, cases positive for PgR were mostly negative for CYP24A1 (p = 0.0485).

The series of 189 tumours with both components (carcinomas *in situ* and the corresponding invasive tumour) allowed the evaluation of the expression of the VDR, CYP27B1 and CYP24A1 simultaneously in the

Table 3 VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in carcinomas *in situ*

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	46 (35.1)	38 (29.0)	58 (42.3)	29 (21.2)	41 (32.8)	36 (28.8)
	- (%)	16 (12.2)	31 (23.7)	33 (24.1)	17 (12.4)	29 (23.2)	19 (15.2)
	p value	**0.0227**		**ns**		**ns**	
HER2	+ (%)	9 (6.9)	14 (10.7)	18 (13.1)	7 (5.1)	9 (7.2)	12 (9.6)
	- (%)	53 (40.5)	55 (42.0)	73 (53.3)	39 (28.5)	61 (48.8)	43 (34.4)
	p value	**ns**		**ns**		**ns**	
PgR	+ (%)	35 (26.7)	30 (22.9)	49 (35.8)	18 (13.1)	38 (30.4)	22 (17.6)
	- (%)	27 (20.6)	39 (29.8)	42 (30.7)	28 (20.4)	32 (25.6)	33 (26.4)
	p value	**ns**		**ns**		**ns**	
CK5	+ (%)	3 (2.3)	8 (6.1)	7 (5.1)	4 (2.9)	8 (6.4)	4 (3.2)
	- (%)	59 (45.0)	61 (46.6)	84 (61.3)	42 (30.7)	62 (49.6)	51 (40.8)
	p value	**ns**		**ns**		**ns**	
EGFR	+ (%)	1 (0.8)	5 (3.8)	2 (1.5)	5 (3.7)	5 (4.0)	3 (2.4)
	- (%)	61 (46.6)	64 (48.9)	89 (65.0)	41 (29.9)	65 (52.0)	52 (41.6)
	p value	**ns**		**0.0295**		**ns**	
P-cad	+ (%)	4 (3.1)	16 (12.2)	14 (10.2)	12 (8.8)	16 (12.8)	7 (5.6)
	- (%)	58 (44.3)	53 (40.5)	77 (56.2)	34 (24.8)	54 (43.2)	48 (38.4)
	p value	**0.0078**		**ns**		**ns**	

ns: not significant.

Table 4 VDR, CYP27B1 and CYP24A1 and other breast cancer biomarkers expression in invasive breast tumours

		VDR		CYP27B1		CYP24A1	
		+ (%)	- (%)	+ (%)	- (%)	+ (%)	- (%)
ER	+ (%)	114 (37.3)	60 (19.6)	70 (25.4)	86 (31.2)	93 (33.1)	66 (23.5)
	- (%)	58 (19.0)	74 (24.2)	53 (19.2)	67 (24.3)	58 (20.6)	64 (22.8)
	p value	**0.0002**		**ns**		**ns**	
HER2	+ (%)	26 (8.6)	34 (11.3)	31 (11.4)	25 (9.2)	29 (10.4)	30 (10.8)
	- (%)	144 (47.7)	98 (32.5)	90 (33.1)	126 (46.3)	121 (43.5)	98 (35.3)
	p value	**0.0238**		**ns**		**ns**	
PgR	+ (%)	71 (23.3)	59 (19.3)	52 (18.8)	64 (23.2)	71 (25.3)	46 (16.4)
	- (%)	100 (32.8)	75 (24.6)	71 (25.7)	89 (32.2)	80 (28.5)	84 (29.9)
	p value	**ns**		**ns**		**0.0485**	
CK5	+ (%)	27 (8.8)	19 (6.2)	15 (5.4)	24 (8.7)	27 (9.6)	16 (5.7)
	- (%)	145 (47.4)	115 (37.6)	108 (39.1)	129 (46.7)	124 (44.1)	114 (40.6)
	p value	**ns**		**ns**		**ns**	
EGFR	+ (%)	4 (1.3)	7 (2.3)	4 (1.5)	6 (2.2)	6 (2.1)	3 (1.1)
	- (%)	166 (54.8)	126 (41.6)	118 (43.1)	146 (53.3)	145 (51.8)	126 (45.0)
	p value	**ns**		**ns**		**ns**	
P-cad	+ (%)	42 (13.8)	40 (13.1)	30 (10.9)	42 (15.2)	40 (14.3)	37 (13.2)
	- (%)	129 (42.3)	94 (30.8)	93 (33.7)	111 (40.2)	110 (39.3)	93 (33.2)
	p value	**ns**		**ns**		**ns**	

ns: not significant.

two types of tumours (Additional file 2: Table S2). The results obtained show that the three proteins (VDR, CYP27B1 and CYP24A1) display a statistically significant correlation of expression between the two sections (carcinomas *in situ* and the matching invasive tumour). Thus, positive cases in the *in situ* component are also positive in the invasive component and the same is observed for the negative cases.

Expression of the VDR, CYP27B1 and CYP24A1 according to the type of breast lesion

The frequencies of protein expression of the VDR, CYP27B1 and CYP24A1 in the different mammary tissues are shown in Figure 2. The normal mammary gland (29 cases), as expected, is positive for the expression of the VDR in all the cases studied (100%). The majority of the samples also displays immunostaining for CYP27B1 (63.6%) and, in contrast, the levels of expression of CYP24A1 are low (29.6%). The VDR is also highly expressed in benign lesions (93.5%) with a reduction in the percentage of positive cases in carcinomas *in situ* (47.3%) and in invasive carcinomas (56.2%). CYP27B1 expression does not vary greatly between the different breast lesions. However, between *in situ* and invasive carcinomas, a statistically significant decrease in the percentage of positive cases was observed (from 66.4% in carcinomas *in situ* to 44.6% in invasive carcinomas). In contrast, the expression of CYP24A1 is increased in carcinomas (56.0% in carcinomas *in situ* and 53.7% in invasive carcinomas) compared with the benign lesions (19.0%), which are mostly negative.

Discussion

Vitamin D mediates anti-proliferative and pro-differentiation signalling in various epithelial tissues, including

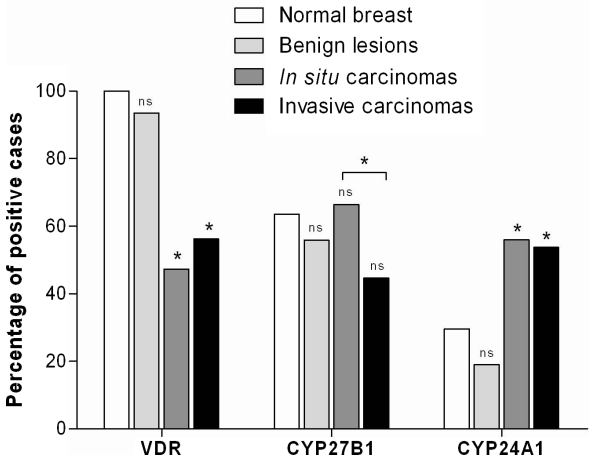

Figure 2 Percentage of positive cases for VDR, CYP27B1 and CYP24A1 in the various types of breast samples studied. Statistical analysis shown use normal breast as reference. An additional result is presented comparing the number of CYP27B1 positive cases between *in situ* and invasive carcinomas. (ns - not significant; * $p < 0.05$).

the mammary gland [6]. Therefore, it is reasonable to assume that disruption of the Vitamin D signalling and metabolic pathways may occur during tumour development. To explore this hypothesis, we have evaluated a cohort of 947 samples of human breast tissues for the presence of VDR, CYP27B1 and CYP24A1. Specifically, our series consisted of normal breast tissue (29 cases), preneoplastic benign mammary lesions (379 cases), carcinomas *in situ* (189 cases) and invasive breast carcinomas (350 cases). To the best of our knowledge, this is the first time that the expression of the VDR, CYP27B1 and CYP24A1 has been evaluated in histological sections of mammary lesions.

The three proteins have been found to be expressed in all breast tissues, although at different levels. VDR presented a nuclear localisation, as it would be expected for a nuclear receptor, while CYP27B1 and CYP24A1 enzymes displayed cytoplasmic staining with a granular pattern, which is consistent with their mitochondrial localisation. The immunohistochemical results were further validated and confirmed using quantitative real-time PCR and Western blotting.

Some studies have demonstrated that the VDR protein is expressed in samples from normal breast tissues and also in breast cancer biopsy specimens [14,15,24,25]. Our results have shown that the VDR is expressed in carcinomas. However, the percentage of positive cases that we have obtained (47.3% in carcinomas *in situ* and 56.2% in invasive carcinomas) is lower than the 80% to 90% that had been previously described in the literature [26,27]. This discrepancy can be explained by the development of new detection techniques and the use of different scoring methods. In this study, we have used the H-Score, the current method employed for other nuclear receptors, like ER [20], whereas in previous studies the presence of any staining was marked as positive. As far as we know, our study is the first to investigate the immunohistochemical expression of the VDR in a range of benign lesions and carcinomas *in situ* of the mammary gland. The percentage of positive cases for the VDR is higher in benign lesions than in invasive tumours (93.5% and 56.2%, respectively), while the carcinomas *in situ* display the lowest value of all (47.3%). There are some studies showing higher levels of VDR in tumour tissues [18,28], but this discrepancy can be attributed to the use of different evaluation techniques.

An interesting finding is the correlation between the expression of the VDR and the ER in both *in situ* and invasive carcinomas. In fact, the VDR is expressed in most ER-positive cases (54.7% in *in situ* carcinomas and 65.5% in invasive tumours). It is thought that one of the VDR functions is to counteract oestrogen-mediated proliferation and maintain differentiation [12]. Indeed, data support the concept that the anti-tumour effects of Vitamin D and its analogues on ER-positive human breast cancer cells are mediated through the down regulation of the ER itself and the attenuation of oestrogen responses, such as breast cancer cell growth [29,30]. Thus, being the VDR mostly expressed in ER-positive carcinomas, Vitamin D or its analogues may become an alternative therapy for these tumours in cases of resistance to ER-targeted therapy.

The levels of protein expression of CYP27B1 and CYP24A1 have not been previously studied in breast cancer. In colon cancer, a study using immunohistochemistry has demonstrated that CYP27B1 is present at equally high levels in normal colonic epithelium and colorectal cancer [31]. For CYP24A1 it has been shown that increasing amounts of this enzyme are present in normal colon tissue and pre-malignant lesions. In cancer, the expression of CYP24A1 decreases as a function of tumour cell dedifferentiation [32]. In breast tissues, McCarthy *et al.*[18] have demonstrated that CYP27B1 mRNA expression was significantly down regulated in adjacent non-cancerous tissue from women with breast cancer in comparison with individuals without cancer. Additionally, it has been shown that the expression of mRNA for CYP27B1 and the VDR was higher in carcinomas versus non-neoplastic tissue [17]. Considering differences in expression in benign and malignant breast tissues, we have observed an increased expression of CYP24A1 and a decreased expression of CYP27B1 with malignant progression. In fact, CYP27B1 was expressed in 55.8% of the preneoplastic lesions and this percentage is decreased in invasive tumours (44.6%), while carcinomas *in situ* display the highest value (66.4%) and these differences are statistically significant. In contrast, CYP24A1 is augmented more than 2.5 fold in invasive tumours (53.7%), compared with benign breast lesions (19.0%) and this difference is also significant ($p < 0.0001$). The *in situ* carcinomas exhibit the highest percentage of positive cases (56.0%). These observations are consistent with the results of Townsend and colleagues [17], which have demonstrated that there was an up regulation of CYP24A1 mRNA in breast tumour tissue, in comparison with normal breast. It has also been described that the *CYP24A1* gene is amplified in breast cancer [33]. In contrast, another study has found no differences in the expression of the VDR, CYP27B1 and CYP24A1 mRNA in breast cancer and non-neoplastic mammary tissue [34]. These contradictory results may be explained by recent reports where it is described that VDR and CYP24A1 are under the post-transcriptional control of miRNAs [35,36].

Breast cancer is a process that evolves through the accumulation of (epi)genetic events that drive uncontrolled proliferation and resistance to apoptosis. The active form of Vitamin D is known for its capacity to modulate proliferation and induce apoptosis [6]. Consequently, malignant cells would need to develop

mechanisms to deregulate Vitamin D metabolic and signalling pathways in order to allow tumour development [37]. Furthermore, it has been suggested that the Vitamin D produced in non-renal tissues is not released into the blood stream, but instead acts locally [38]. Therefore, the amount of Vitamin D available in the tissue depends on the relative amounts of CYP27B1 (synthesis) and CYP24A1 (catabolism). Accordingly, our results show a deregulation of these two enzymes in the different stages of breast carcinogenesis. The crucial step of transformation introduces a clear unbalance in the Vitamin D signalling and metabolic pathways. A reduction in the expression of the VDR in carcinomas indicates lower sensitivity of the tissue to Vitamin D control. Furthermore, a strong increase in CYP24A1 positive cases points to an enhanced ability of the cells to degrade this hormone. In contrast, the stable levels of CYP27B1 throughout the transformation process, with only a small decrease in invasive carcinomas, may reflect a lower capacity to metabolize Vitamin D into its active form.

Conclusions

In summary, this is the first study to report the expression of the VDR, CYP27B1 and CYP24A1 in a series of normal breast, preneoplastic mammary lesions, breast carcinomas *in situ* and invasive tumours. We have correlated the expression of these Vitamin D partners with the expression of a panel of tumour biomarkers. Furthermore, we have confirmed these results by real-time RT-PCR. Overall, our results on the expression of the VDR, CYP27B1 and CYP24A1 suggest that there is a deregulation of the Vitamin D metabolic and signalling pathways in breast cancer, in order to favour tumour progression. Thus, during breast malignant transformation, tumour cells lose their ability to synthesize the active form of Vitamin D and to respond to Vitamin D effects, while increasing their ability to degrade this hormone.

Additional material

Additional file 1: Figure S1: In MDA-MB-231 breast cancer cells CYP27B1 expression is induced by the treatment with PTH 100 nM for 4 h and CYP24A1 expression is induced by the treatment with Vitamin D ($1,25(OH)_2D_3$) 100 nM for 72 h. α-tubulin was used as a loading control

Additional file 2: Table S2: VDR, CYP27B1 and CYP24A1 expression in tumours that display both the *in situ* and the invasive component in the same histological section.

List of abbreviations

VDR: Vitamin D Receptor; TMA: Tissue Microarray; ER: oestrogen receptor; CK: Cytokeratin; EGFR: Epidermal Growth Factor Receptor; UDH: Usual Ductal Hyperplasia; ADH: Atypical Ductal Hyperplasia; CCL: Columnar Cell Lesions

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NL performed the practical work, analysed the data and drafted the manuscript. BS, DM and MG participated in the practical work. DV, LAV and FM analysed the data. JP and JLC designed the study and contributed to the manuscript. FS conceived the study, participated in its design and coordination, analysed the data and contributed to the manuscript. All authors read and approved the final manuscript.

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Paper VI

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Histology and Histopathology

Cellular and Molecular Biology

P-cadherin, Vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study

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Summary. Introduction: The most suitable immunohistochemical criterion to identify basal-like breast carcinomas (BLBC), a molecular subgroup of breast cancer associated with poor prognosis, is the triple negative phenotype along with CK5 and/or EGFR immunoreactivity. However, several putative basal markers have been suggested as alternatives to identify BLBC with more accuracy. Experimental Design: The expression of CK5, EGFR, P-cadherin, CK14, Vimentin and p63 were evaluated in 462 invasive breast carcinomas to determine their sensitivity and specificity for BLBC identification. Results: P-cadherin and CK5 showed higher sensitivity values, while EGFR, Vimentin and CK14 were the most specific markers. The combination of CK5 with P-cadherin, Vimentin or CK14 proved to be a reliable option for distinguishing the basal phenotype, compared to the "gold standard" pair CK5/EGFR. Furthermore, P-cadherin was still able to recognize a large number of putative BLBC among the "unclassified" group (ER-/PR-/HER2-/CK5-/EGFR-). Conclusions: P-cadherin, Vimentin and CK14 can recognize BLBC already identified in triple negative/ CK5 and/or EGFR+ tumors, and due to P-cadherin sensitivity for BLBC identification this marker can reliably recruit a large number of breast carcinomas with basal phenotype among immunohistochemistry triple negative/ CK5 and/or EGFR - pool of tumors. Although they need GEP validation, our results can introduce the idea of these markers as additional options in the daily workup of breast pathology laboratories to identify BLBC.

Key words: Basal-like breast cancer, P-cadherin, CK14, Vimentin

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Introduction

In the European Union, breast cancer is the most incident form of cancer in women, with an estimated 429.900 cases diagnosed per year (28.9% of all incident cases in women) (Ferlay et al., 2007; Milanezi et al., 2008). Breast cancer is frequently designated as a heterogeneous disease with divergent biological behaviors. cDNA microarray studies have provided an improvement in cellular and molecular understanding of breast cancer, identifying distinct subtypes of breast carcinomas with different molecular signatures and clinical outcomes (Perou et al., 2000; Sorlie et al., 2001, 2003; Rakha et al., 2006a,b). The basal-like subtype has definitely drawn the attention of the scientific community. These tumors are characterized by a triple negative (TN) phenotype, lacking the expression of hormone receptors (HR) [estrogen and progesterone receptors (ER and PR, respectively)] and HER2. Basal-like breast carcinomas (BLBC) are associated with

aggressive tumor behavior and shorter overall survival when compared to the luminal and HER2-overexpressing subtypes and there is an enthusiastic search for molecular markers expressed in BLBC that could be used as targets to therapy (Nielsen et al., 2004). Histologically, they are poorly differentiated carcinomas, present high nuclear and histological grade and frequently show medullary and metaplastic features (Tsuda et al., 2000; Fulford et al., 2006; Livasy et al., 2006; Rakha et al., 2006a,b). A distinct pattern of metastasis to brain and lungs, known to be associated with poor prognosis, and less significant involvement of axillary lymph nodes, has also been described in BLBC (Tsuda et al., 2000; Banerjee et al., 2006; Fulford et al., 2007). Nowadays, gene expression profiles (GEP) or cDNA microarrays studies are currently considered the "gold standard" methods for the identification of breast carcinomas with basal phenotype, since these technologies were the first to identify BLBC as a distinct subgroup with a specific molecular signature (Perou et al., 2000) and clinical identity (Sorlie et al., 2001, 2003; van't Veer et al., 2002). However, GEP are expensive, not easily applicable as a routine laboratory diagnostic tool in large scale clinical-pathological analysis and have limited value in retrospective studies using formalin-fixed paraffin-embedded (FFPE) tissues (Cheang et al., 2008; Reis-Filho and Tutt, 2008). Thus, the idea of developing an immunohistochemical (IHC)-based assay for the identification of BLBC is appealing. The variation in the transcriptional and translational programs of cells that accounts for the different molecular identities of breast carcinomas also reinforces the interest in creating an IHC-based assay for BLBC definition. The characteristic protein expression of tumors would be a useful surrogate of GEP, and the IHC profile would help to standardize investigations and uniformly identify a group of tumors with a basal-like transcriptional program (Reis-Filho and Tutt, 2008).

However, the most appropriate panel of antibodies to be used, in order to identify breast carcinomas with basal phenotype, has not reached a consensus yet. In 2008, Tang et al. (2008) compared the different IHC classifications that have been used to define basal-like and non basal-like breast carcinomas; interestingly, they showed that in high grade breast carcinomas, which is a common feature of basal phenotype, the rates of BLBC ranged between 19% and 76%, indicating the need for a more consensual strategy between laboratories.

The TN phenotype criterion is used by some authors who assume that Triple Negative tumors and BLBC are synonymous (Kreike et al., 2007; Spitale et al., 2008). In fact, this criterion is quite convenient, since it includes standard biomarkers already used in the clinical management of breast cancer. However, relying on negative results to perform a diagnostic interpretation may be risky due to technical failures leading to a decrease in specificity. Other authors use high molecular weight cytokeratins alone (CK5/6, CK14 or CK17) to identify BLBC, claiming that BLBC and triple negative tumors are different identities (van de Rijn et al., 2002; Abd El-Rehim et al., 2004; Fulford et al., 2007; Rakha et al., 2007b). In addition, since basal-like breast carcinomas express proteins that are characteristic from the basal/myoepihelial outer layer of the mammary gland, such as EGFR, p63, P-cadherin, calponin, CD10, S100 and α-smooth-muscle actin (α-SMA) (Jones et al., 2001; Reis-Filho et al., 2003; Nielsen et al., 2004; Livasy et al., 2006), some definitions of BLBC associate the lack of expression of ER, PR and HER2 with the immunoreactivity for some of these basal markers that were already correlated with basal phenotype and poor prognosis (Nielsen et al., 2004; Matos et al., 2005; Laakso et al., 2006). Our group has previously demonstrated that using a panel of antibodies for ER, PR, HER2, CK5/6 and/or EGFR and/or P-cadherin and/or p63 it is possible to distinguish invasive (Matos et al., 2005) and i*n situ* (Paredes et al., 2007b) BLBC. However, Nielsen et al. (2004) found that expression of CK5/6 and EGFR together with negativity for ER and HER2 would be the immunoprofile that identifies the same basal-like carcinomas found by cDNA microarrays, with a sensitivity of 76% and a specificity of 100%. This criterion is, therefore, considered the "gold standard" immunoprofile to classify BLBC.

In this study, we aim to refine the immunohistochemical criterion to identify BLBC by analyzing the sensitivity and the specificity of the main basal markers that have been described, namely CK5, EGFR, P-cadherin, CK14, Vimentin and p63 and suggest possible additional markers for BLBC identification, especially in CK5 and EGFR negative breast carcinomas.

Materials and methods

Breast tumour samples

Formalin-fixed, paraffin-embedded tissues of 462 invasive breast carcinomas were consecutively retrieved from the histopathology files of three Departments of Pathology: University Hospital of the Federal University of Santa Catarina (Florianópolis, Brazil), Hospital Divino Espírito Santo (HDES), (Ponta Delgada, São Miguel, Portugal), and a private Laboratory of Pathology in Araçatuba, Brazil. All cases were reviewed by three pathologists (FM, FS and LV) on haematoxylin and eosin-stained (H&E) sections.

TMA construction

Representative areas of the invasive breast carcinomas were carefully selected on the H&E-stained sections and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each specimen and precisely deposited into a recipient paraffin block using a TMA workstation (TMA builder 20010.02, Histopatholoy Ltd, Hungary). Forty seven TMA blocks were constructed, each one containing 24 tissue cores, arranged in a 4x6 sector. In each TMA

block, normal breast and testicular tissue were included as controls. After construction, 2 μm tissue sections were cut and adhered to glass slides (Polysine*TM*, Menzel-Glasser, Germany) for the immunohistochemical studies and a H&E-stained section from each TMA block was reviewed in order to confirm the presence of morphological representative areas of the original lesions.

Immunohistochemistry

All the immunohistochemical assays were performed with specific monoclonal antibodies. Details about primary antibodies, antigen retrieval and IHC detection systems are described in Table1. Except for EGFR, in which epitope retrieval was performed by proteolytic enzyme digestion for 20 minutes (pepsin A, 4 g/l; Sigma-Aldrich, USA) at 37°C, all epitope retrieval was heat-induced at 98ºC in a water-bath during 30 minutes, using a commercially available citrate buffer solution (Vector Laboratories, USA), 1:100, pH=6.0, or an ethylenediaminetetraacetic (EDTA) solution (Novocastra, UK), 1:10, pH=9.0, as antigen unmasking solutions. After the respective antigen retrieval and washes in a phosphate buffer solution (PBS), endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution (Panreac, Spain) in methanol (Sigma-Aldrich, USA) for 10 minutes. The slides were incubated in a blocking serum (LabVision, USA) for 15 min and then incubated with the respective primary monoclonal antibodies. Immunoassays were performed using the streptavidin-biotin-peroxidase technique (SABC), (LabVision Corporation, Fremont, CA, USA) or the HRP labeled polymer (DakoCytomation, USA) detection system, according to manufacturer's instructions. All reactions were revealed with diamino-benzidine (DAB) chromogen (DakoCytomation). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and coverslipped using a permanent mounting solution (Mounting Medium, Richard Allan Scientific, USA). Positive and negative controls were included in every set of reactions for each antibody used. Normal breast ducts and lobules present in many of the selected areas were also used as internal controls, as well as the non-neoplastic breast tissue cores included in each array. The evaluation of immunohistochemistry results was performed by three pathologists as follows: ER, PR and p63 were considered positive whenever more than 10% of the neoplastic cells showed nuclear staining; similarly, the same cutoff was used for CK5, CK14 and Vimentin cytoplasmic staining, as well as for P-cadherin membrane staining. Membrane expression for HER2 and EGFR was evaluated according to the DakoCytomation HercepTest® scoring system (Reis-Filho et al., 2005). Breast carcinomas were considered HER2-overexpressing whenever the immunohistochemical reaction was classified as 3+ or when gene amplification was confirmed by Chromogenic *In Situ* hybridization (CISH) in the 2+ cases, as described in other works (Ricardo et al., 2007). For EGFR, the cases were considered positive whenever the immunostaining was 2+ or 3+.

Hormone receptor (ER and PR) positive tumors were considered luminal A and B whether or not they overexpressed HER2, respectively (Sotiriou et al., 2003; Matos et al., 2005; Paredes et al., 2007b; Spitale et al., 2008; Tamimi et al., 2008). Cases lacking ER/PR with overexpression of HER2 were classified as HER2 overexpressing tumors. ER-/PR-/HER2- cases with immunoreactivity for EGFR and/or CK5 were considered BLBC according to the gold standard Nielsen's criterion and cases without expression of the five biomarkers were considered unclassified. When the immunoreactivity for the additional basal markers, namely P-cadherin, CK14 and Vimentin are used, the positive cases for at least one of these markers were considered as BLBC (P-cad and/or CK14 and/or Vim). Since for some markers the immunohistochemical result was not interpretable, the statistical analyses were performed using only 387 breast tumors cases which were classified for all the biomarkers tested.

Table 1. Conditions of the immunohistochemical reactions performed in this study.

	Primary antibodies				Antigen retrieval buffer	Detection method
Antigen	Clone	Origin	Incubation time (min)	Dilution		
ER	SP1	Neomarkers, USA	30	1:150	Citrate	SABC*
PR	SP2	Neomarkers, USA	30	1:300	Citrate	HRP-Polymer **
HER2	SP3	Neomarkers, USA	30	1:80	Citrate	SABC*
CK5	XM26	Neomarkers, USA	60	1:50	Tris-EDTA	SABC*
EGFR	31G7	Zymed	60	1:100	Pepsin	HRP-Polymer **
P-cadherin	56	BD Transduction	60	1:50	Tris-EDTA	HRP-Polymer **
CK14	LL002	Novocastra, UK	60	1:400	Tris-EDTA	HRP-Polymer **
Vimentin	V9	Dako, USA	30	1:150	Citrate	SABC*
p63	4A4	Neomarkers, USA	60	1:150	Citrate	SABC*

* SABC: streptavidin-avidin-biotin-complex; **: HRP-Polymer (horseradish peroxidase - polymer).

Statistical analysis

Statistical analysis was performed by SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA) software program. χ^2 contingency test was used to determine associations between groups and the results were considered statistically significant if the p value was lower than 0.05. In order to determine which were the most sensitive and specific biomarkers to identify BLBC, the sensitivity and the specificity of the antibodies used were calculated. Sensitivity measurement was defined by the quotient between the true positive (TrueP) cases and the sum of the true positive and the false negative (FalseN) cases [sensitivity = TrueP/(TrueP+FalseN)]. Specificity was measured in a similar way, by the quotient between the true negative (TrueN) cases with the sum of the true negatives and the false positives (FalseP) [specificity = TrueN/(TrueN+FalseP)]. PPV (Positive Predictive Value) and NPV (Negative Predictive Value) were calculated as follows: PPV = TrueP/(TrueP+FalseP) and PNV = TrueN/(TrueN+FalseN). As described before, ER/PR/HER2 negative tumors that express CK5/6 and/or EGFR were considered BLBC. Consequently, TrueP and TrueN cases were the BLBC tumors that were positive or negative, respectively, to the marker or pair of markers in analysis. Inversely, FalseP and FalseN were non BLBC positive or negative to the basal markers in study.

Follow-up information was available for 282 of the 387 cases and a maximum cutoff of 77 months was considered. Survival curves were estimated by the Kaplan-Meier method using log-rank test to assess significant differences for overall survival.

Results

In this series of 387 breast carcinomas, 223/387 (57.6%) and 144/387 (37.2%) cases were ER and PR positive, respectively, and 65/387(16.8%) overexpressed HER2. Using the ER/PR/HER2- (TN) criterion, this series comprises 109 (28.2%) triple negative and 278 (71.8%) non-Triple Negative tumors. Considering the molecular subtypes of breast cancer, 213 (55%) cases were luminal A, 13 (3.4%) luminal B and 52 (13.4%) HER2-overexpressing tumors. According to Nielsen's criterion, 37 (9.6%) cases presented a basal-like phenotype and 72 (18.6%) were considered "unclassified" by this criterion. We analyzed the associations between CK5, EGFR, P-cadherin, CK14, p63 and Vimentin and the BLBC versus non BLBC (Table 2). As expected, the markers were significantly associated with the basal phenotype ($p<0.0001$), with the exception for p63 ($p=0.5403$). Fig. 1 shows the immunohistochemical staining for CK5, EGFR, P-cadherin, Vimentin and CK14 in BLBC.

Afterwards, the sensitivity, specificity, PPV and NPV of each biomarker for the identification of BLBC were calculated (Table 3), except for p63 which was not even related with basal phenotype. CK5 was the most sensitive biomarker (91.9%), followed by P-cadherin (67.6%). CK14 and EGFR were the most specific markers, presenting 98.6% and 97.1% of specificity, respectively, and vimentin was also shown to be very specific (86.9%).

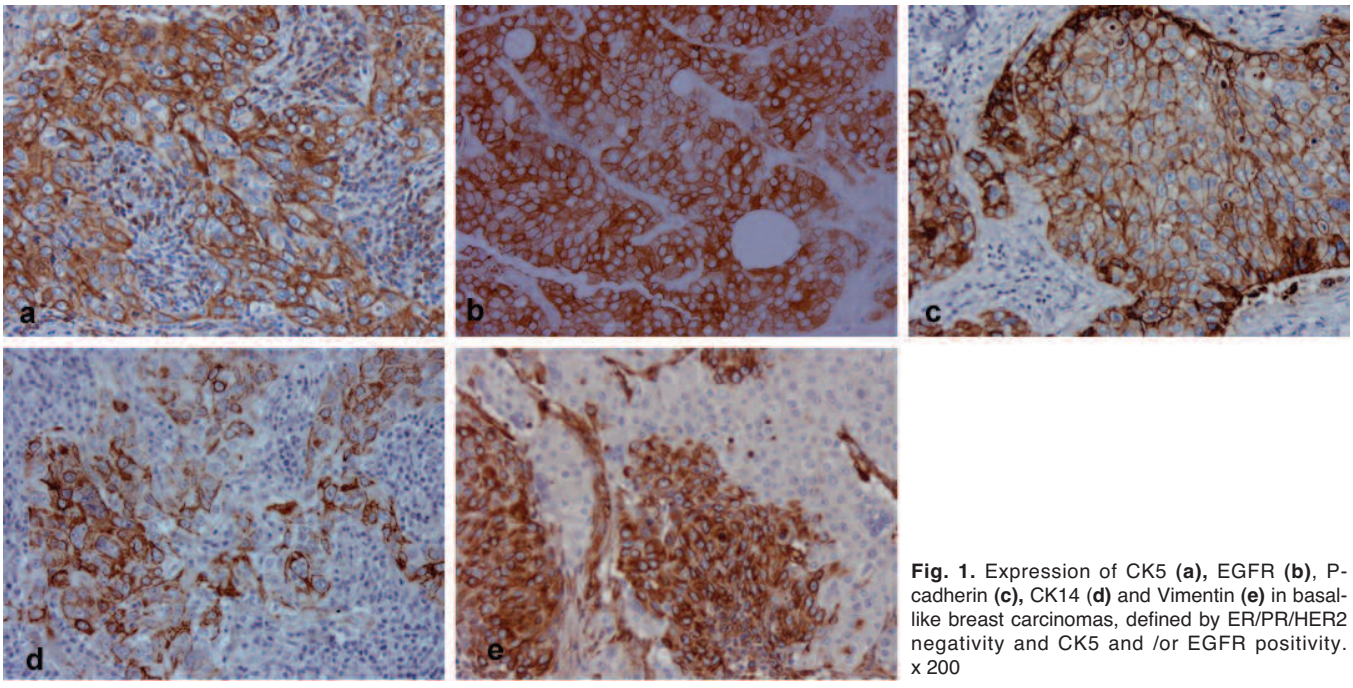

Fig. 1. Expression of CK5 **(a),** EGFR **(b)**, P-cadherin **(c),** CK14 **(d)** and Vimentin **(e)** in basal-like breast carcinomas, defined by ER/PR/HER2 negativity and CK5 and /or EGFR positivity. x 200

In order to find the best combination of basal markers with the ability to identify BLBC, we evaluated the most sensitive and the most specific markers in pairs (CK5, P-cadherin with CK14, EGFR or Vimentin). Since P-cadherin presented good sensitivity and specificity values, we also evaluated its association with CK5 (Table 4). The statistical associations considered cases that were positive for both markers (+/+), positive for at least one marker (+/- or -/+) or negative for both (-/-). Table 5 shows the percentages of sensitivity, specificity, PPV and NPV for the several pairs of markers. In these analyses, we considered as true positive the cases that were +/+ and positive for at least one of the markers in the subgroup of BLBC previously distinguished by Nielsen's criterion, and as false positive the cases that

Table 2. Association between the expression of CK5, EGFR, P-cadherin, CK14, p63 and vimentin with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	P
	387	37(9.6%)	350(90.4%)	
CK5				<0.0001
+	89	34(91.9%)	55(15.7%)	
-	298	3(8.1%)	295(84.3%)	
EGFR				<0.0001
+	21	11(29.7%)	10(2.9%)	
-	366	26(70.3%)	340(97.1%)	
P-cadherin				<0.0001
+	123	25(67.6%)	98(28%)	
-	264	12(32.4%)	252(72%)	
CK14				<0.0001
+	17	12(32.4%)	5(1.4%)	
-	370	25(67.6%)	345(98.6%)	
p63				0.5403
+	14	2(5.4%)	12(3.4%)	
-	373	35(94.6%)	338(96.6%)	
Vimentin				<0.0001
+	63	17(45.9%)	46(13.1%)	
-	324	20(54.1%)	304(86.9%)	

Table 3. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the IHC method for the basal-markers studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5	91.9	84.3	38.2	99.0
EGFR	29.7	97.1	52.4	92.9
P-cadherin	67.6	72.0	20.3	95.5
CK14	32.4	98.6	70.6	93.2
Vimentin	45.9	86.9	27.0	93.8

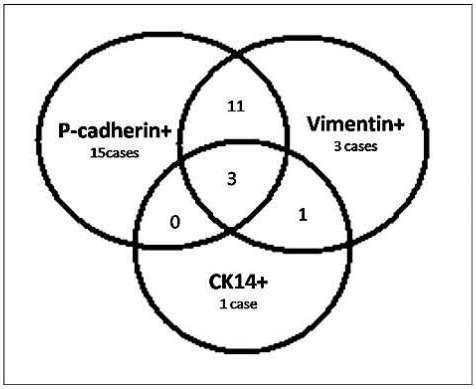

Fig. 2. Distribution of P-cadherin, vimentin and CK14 expression in triple negative tumors that were negative for CK5 and EGFR.

Table 4. Association between the expression of pairs of basal markers with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	p
CK5/EGFR				<0.0001
+/+	11	8(21.6%)	3(0.8%)	
At least one +	88	29(78.4%)	59(16.9%)	
-/-	288	0(0%)	288(82.3%)	
CK5/CK14				<0.0001
+/+	11	11(29.7%)	0(0%)	
At least one +	83	23(62.2%)	60(17.1%)	
-/-	293	3(8.1%)	290(82.9%)	
CK5/Vim				<0.0001
+/+	24	16(43.2%)	8(2.3%)	
At least one +	104	19(51.4%)	85(24.3%)	
-/-	259	2(5.4%)	257(73.4%)	
P-cadherin/EGFR				<0.0001
+/+	13	8(21.6%)	5(1.4%)	
At least one +	118	20(54.1%)	98(28%)	
-/-	256	9(24.3%)	247(70.6%)	
P-cadherin/CK14				<0.0001
+/+	12	9(24.3%)	3(0.9%)	
At least one +	116	19(51.4%)	97(27.7%)	
-/-	259	9(24.3%)	250(71.4%)	
P-cadherin/Vim				<0.0001
+/+	41	11(29.7%)	30(8.6%)	
At least one +	104	20(54.1%)	84(24%)	
-/-	242	6(16.2%)	236(67.4%)	
P-cadherin/CK5				<0.0001
+/+	38	23(62.2%)	15(4.3%)	
At least one +	136	13(35.1%)	123(35.1%)	
-/-	213	1(2.7%)	212(60.6%)	

Table 5. Sensitivity, specificity, PPV and NPV of the IHC method for the pairs of basal-markers antibodies studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5/EGFR	100	82.3	11.4	100
CK5/CK14	91.9	82.9	10.5	99
CK5/Vim	94.6	73.4	12.0	99.2
P-cadherin/EGFR	75.7	70.6	10.2	96.5
P-cadherin/CK14	75.7	71.4	10.1	96.5
P-cadherin/Vim	83.8	67.4	11.6	97.5
P-cadherin/CK5	97.3	60.6	14.5	99.5

were positive for the two markers and the ones expressing at least one marker in non basal-like tumors. True negative and false negative were the -/- cases in non basal-like and in BLBC, respectively. All the associations were statistically significant ($p<0.0001$). The pair CK5/EGFR presented, as expected, the highest values of sensitivity and specificity, 100% and 82.3%, respectively. However, concerning sensitivity, the pairs CK5/CK14, P-cadherin/CK5 and CK5/Vimentin showed similar values to the "gold standard" CK5/EGFR pair, with 91.9%, 97.3% and 94.6% of sensitivity, respectively. The specificity of CK5/CK14 combination (82.9%) was approximately equal to the one presented by CK5/EGFR (82.3%).

In the BLBC group, when analyzing the number of cases that were +/+ and positive for at least one of the markers of the pair, against the -/- cases (Table 6), it is possible to observe that only one basal-like breast carcinoma was negative for both markers in P-cadherin/CK5 pair. The CK5/Vimentin pair missed the expression in 2 cases, while CK5/CK14 did not stain three BLBC. All the other pairs were positive in BLBC for the two markers, or for at least one of them, in at least 75.7% of breast carcinomas with basal phenotype.

More importantly, given the sensitivity of P-cadherin and the specificity of CK14 and Vimentin, we also analyzed their expression among the TN/CK5 and EGFR

Table 6. Analyzes of the distribution of expression of the pairs of markers in BLBC.

		Basal n (%)
CK5/EGFR	+/+ and at least one + -/-	37(100%) 0(0%)
CK5/CK14	+/+ and at least one + -/-	34(91.9%) 3(9.1%)
CK5/Vim	+/+ and at least one + -/-	35(94.6%) 2(5.4%)
P-cadherin/EGFR	+/+ and at least one + -/-	28(75.7%) 9(24.3%)
P-cadherin/CK14	+/+ and at least one + -/-	28(75.7%) 9(24.3%)
P-cadherin/Vim	+/+ and at least one + -/-	31(83.8%) 6(16.2%)
P-cadherin/CK5	+/+ and at least one + -/-	36(97.3%) 1(2.7%)

Table 7. Expression of P-cadherin, vimentin and CK14 in the 72 TN tumors also negative for CK5 and EGFR.

		TN/CK5 and EGFR- n=72
P-cadherin	+ -	29(40.3%) 43(59.7%)
Vimentin	+ -	18(25%) 54(75%)
CK14	+ -	5(6.9%) 67(93.1%)

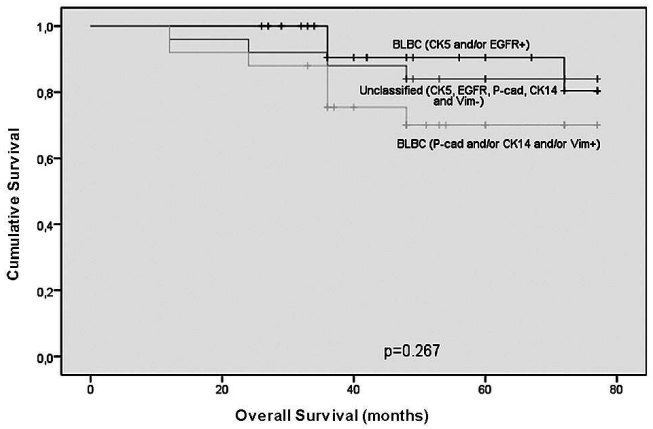

Fig. 3. Kaplan-Meier survival curves for overall survival (OS) of triple negative breast carcinoma patient's cohort, with a 77 months cut-off. BLBC defined by TN/CK5 and/or EGFR+ [BLBC (CK5 and/or EGFR+)], BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin [BLBC (P-cad and/or CK14 and/or Vim)] and tumors that were negative for all the basal markers in study were analyzed, p=0.267 (not statistically significant).

Table 8. Distribution of histological grade among triple negative breast carcinomas of the studied series.

	Histological grade		
Triple negative tumors (n=103*)	I	II	III
BLBC (CK5 and/or EGFR+) (n=34)	3 (9%)	12 (35%)	19 (56%)
BLBC (P-cadherin and/or CK14 and/or Vimentin+) (n=32)	2 (6%)	15 (47%)	15 (47%)
Unclassified (TN,CK5, EGFR, P-cad, CK14 and Vim-) (n=37)	17 (46%)	15 (40%)	5 (14%)

BLBC (CK5 and/or EGFR+) are the TN tumors that were positive for CK5 and/or EGFR and BLBC (P-cadherin and/or CK14 and/or Vimentin+) are the TN/CK5 and EGFR- tumors immunoreactive for one of the additional markers in study: P-cadherin, CK14 and vimentin. *: Histological grade of some cases could not be assessed because the patients were submitted to preoperative chemotherapy.

negative tumors ("unclassified" by Nielsen's criterion). In 38/72 (52.8%) cases, none of the biomarkers were expressed; however, in the other 34/72 cases (47.2%), there was the expression of, at least, one of the biomarkers. P-cadherin was present in 29 (40.3%), Vimentin in 18 (25%) and CK14 in 5 (6.9%) of these tumors (Table 7). In a more detailed analysis, 15 cases were positive only for P-cadherin, while only one and three cases were positive for CK14 and for Vimentin alone, respectively (Fig. 2).

Interestingly, if we consider as BLBC these TN/CK5 and EGFR- "unclassified" cases that presented immunoreactivity for P-cadherin, CK14 and/or Vimentin [BLBC (Pcad and/or CK14 and/or Vimentin+)], this series presents 71/387 (18%) of BLBC. BLBC defined by TN/CK5 and/or EGFR+ and BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin were analyzed separately. These two differently defined BLBC presented a similar percentage of high histological grade tumors [56% and 47% in BLBC (CK5 and/or EGFR+) and in BLBC (Pcad and/or CK14 and/or Vimentin+), respectively], (Table 8). The overall survival was similar for the two groups as we can see in Figure 3.

Discussion

The need for a more precise diagnosis of breast cancer that converges with the clinical outcome and the choice of the most appropriate therapy has motivated studies in different areas of breast cancer research. The cDNA microarray technology is a "gold standard" method for the recognition of the basal phenotype, but from a practical point of view, we need to translate these results to an accessible method. It is undeniable that the BLBC immunohistochemistry definition requires cDNA microarray validation, since these tumors were first identified by this technique (Perou et al., 2000; Livasy et al., 2006). However, from the pathologists and oncologists point of view, the lack of molecular targets for therapy in this subgroup of patients indicates the urgent need for an easier and less expensive way to identify BLBC patients. Based on this, there is an attempt to establish an immunohistochemical surrogate panel, easily applied on FFPE samples, which identifies a pool of breast cancer patients who may require more aggressive systemic therapy and that would be the most appropriate subjects for clinical trials, specifically targeting this molecular subgroup of breast cancer. However, there is still no consensual definition about the ideal IHC panel of biomarkers to distinguish the basal phenotype. In fact, many different panels have been used, in which CK5, EGFR, P-cadherin, CK14 and Vimentin are included. Due to this diversity of criteria, a wide range of percentages of BLBC are described in the several studied series (van de Rijn et al., 2002; Foulkes et al., 2004; Jones et al., 2004; Abd El-Rehim et al., 2005; Arnes et al., 2005; Collett et al., 2005; Kusinska et al., 2005; Laakso et al., 2005; Potemski et al., 2005; Banerjee et al., 2006; Fulford et al., 2006, 2007; Kim et al., 2006; Rakha et al., 2006a,b, 2007a,b,c; Rodriguez-Pinilla et al., 2006, 2007; Siziopikou and Cobleigh, 2007). Nielsen et al. (2004) demonstrated that CK5 and EGFR could reliably discriminate BLBC that were identified by GEP, considering these two basal markers the "gold standard" immunohistochemical panel of antibodies to the BLBC identification, together with ER and HER2 lack of expression. Recently, Cheang et al. (2008) compared two BLBC immuno-panels and concluded that the ER-/PR-/HER2- and expression of CK5 and/or EGFR provides the more accurate definition of BLBC and can better predict breast cancer patient's survival.

However, we cannot assure which are the best antibodies to be included in a daily practice panel for the recognition of the basal phenotype in breast carcinomas: should we look for the most sensitive or the most specific ones? None of these markers are actually pathognomonic of a basal phenotype, since they are variably expressed in the other subgroups of breast carcinomas, which support the search for "ideal" biomarkers to be used in the anatomic pathology workup and with clinical relevance.

We demonstrate herein that P-cadherin, Vimentin or CK14 may possibly be useful biomarkers to include in IHC panels for distinguishing BLBC. P-cadherin reveals consistent values of sensitivity and specificity, while Vimentin and CK14 presented high specificity values. The three markers were able to reliably recognize the basal phenotype, especially when associated to CK5.

The presence of P-cadherin, an adhesion molecule expressed in myoepithelial cells of the normal mammary gland, was already described in invasive and in *in situ* breast carcinomas with worst prognosis, namely in those with high histological grade and basal phenotype (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005, 2007b). The role of P-cadherin in breast carcinogenesis has been one of the main fields of our research group's interest and we have observed that this molecule presents an inverse correlation with HR (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005) and a direct correlation with EGFR (Kovacs and Walker, 2003), HER2 and high proliferation rates, strengthening the value of P-cadherin as a poor prognostic indicator in breast cancer (Palacios et al., 1995; Peralta Soler et al., 1999; Gamallo et al., 2001; Paredes et al., 2005). The expression of P-cadherin in neoplastic cells has already been related to a histogenetic origin in cap cells or to the acquisition of a stem cell-like phenotype, suggesting that P-cadherin-expressing tumors could be associated to a stem cell origin (Peralta Soler et al., 1999, Gamallo et al., 2001, Paredes et al., 2007). Recently, it has been suggested that basal-like breast carcinomas may be genuine stem/early progenitor cell tumors of the mammary gland, relating their origin to a more undifferentiated type of precursor cells (Honeth et al., 2008). Also, Rakha et al. (2009)

demonstrated more evidence of the features of dual-lineage differentiation/stem cell phenotype of BLBC by showing a higher frequency of CK19 expression in this type of tumor.

CK14 does not show a differential presence in breast carcinomas with basal phenotype identified by cDNA microarray technology, but this cytokeratin is frequently associated with poor prognosis (Jones et al., 2004) and with the morphological features observed in BLBC (Tsuda et al., 2000). For this reason, CK14 has been included in the immunopanel used to identify BLBC by several other authors (Laakso et al., 2005, 2006; Rakha et al., 2006a,b; Reis-Filho et al., 2006).

Vimentin is an intermediate filament protein whose expression in normal mammary gland is also restricted to myoepithelial/ basal layer. Its expression has been associated with high histological grade, lack of ER, p53 mutations, high proliferation rates (Raymond and Leong, 1989; Domagala et al., 1990a,b; Koutselini et al., 1995; Santini et al., 1996; Thomas et al., 1999) and expression of CK5/6 and EGFR (Korsching et al., 2005; Reis-Filho, 2005). Vimentin-expressing carcinomas have been observed in association with sporadic and familial BLBC and with a specific pattern of metastasis similar to BLBC (Rodriguez-Pinilla et al., 2007). Like P-cadherin, Vimentin was also described to be differentially expressed by BLBC identified by GEP, being proposed to integrate the panel of antibodies for the identification of BLBC (Livasy et al., 2006).

Our results show that P-cadherin, CK14 and Vimentin, together with CK5, can identify almost all BLBC that were classified as such using the most widely accepted IHC panel to classify BLBC: ER/PR/HER2- and CK5 and/or EGFR+.

Triple negative phenotype by IHC is one of the characteristic features of BLBC and several authors claim that basal tumors are almost all TN tumors (Diaz et al., 2007; Kreike et al., 2007). Kreike et al. (2007), in a series of 97 TN cases, observed that 90% of these tumors have a basal phenotype by cDNA microarray analysis. However, the lack of expression of ER, PR and HER2 as the sole criterion to identify these tumors is risky (Rakha et al., 2008) because there are technique limitations when dealing with FFPE tissue samples, which reinforces the need for a more suitable panel.

There is a significant overlapping of features shared by triple negative and BLBC in what concerns, for example, the prevalence of these types of cancer in younger patients, in African-American women (Morris et al., 2007), their presentation as interval cancers, a similar pattern of recurrence (Dent et al., 2007; Tischkowitz et al., 2007), the more aggressive behavior comparing with other types of breast cancer (Reis-Filho and Tutt, 2008) and the biological and clinical similarity between sporadic TN and BLBC with breast carcinomas arising from BRCA1 mutation carriers (Reis-Filho and Tutt, 2008). However, several studies claim that this overlap is not complete (Bertucci et al., 2008, Rakha and Ellis, 2009). It is known that TN carcinomas with basal phenotype have a significant shorter disease-free survival than TN without expression of basal markers (Rakha et al., 2007a; Tischkowitz et al., 2007) and that germline BRCA1 mutation carriers are more probably found in TN tumors expressing CK5/6 and /or EGFR than in TN with no expression of these basal markers (Turner et al., 2007; Rakha et al., 2009). It has also been observed in GEP that triple negative group is composed by other subgroups of tumors with different outcomes, namely the normal breast-like tumors (Perou et al., 2000; Sorlie et al., 2001, 2003; Sotiriou et al., 2003; Fan et al., 2006; Hu et al., 2006; Hennessy et al., 2009) and a recently described subgroup of claudin-low tumors (Herschkowitz et al., 2007; Hennessy et al., 2009). The existence of TN tumors that do not react immunohistochemically with any of the basal markers routinely used has been described, and variably designated as non basal triple negative, unclassified, undetermined, null phenotype (Liu et al., 2008) or TN3BKE- (Triple Negative 3 Basal Keratins and EGFR-) (Rakha et al., 2009). It seems extremely important to distinguish BLBC from the whole triple negative group, reducing the TN heterogeneity, since their biological behavior appears to be different. The lightening of this heterogeneity would enable patients to benefit from their differential recognition (Rakha et al., 2007a, 2008, 2009; Liu et al., 2008; Reis-Filho and Tutt, 2008; Tan et al., 2008; Rakha and Ellis, 2009). This distinction is also important because TN tumors defined by IHC tend to be clinically considered as BLBC and selected for clinical trials (Bertucci et al., 2008), probably misleading the effect of the drugs in the clinical trials.

It is interesting to emphasize that among the analyzed TN/CK5 and EGFR- tumors that were also negative for P-cadherin, CK14 and Vimentin, approximately 50% of these cases presented low histological grade (Table 8). P-cadherin was expressed alone in a higher number (15 cases) of TN/CK5 and EGFR negative tumors, compared with CK14 (1 case) and Vimentin (3 cases). When P-cadherin, CK14 and Vimentin expression are considered along with CK5 and EGFR for the BLBC identification, 34 cases are added to the 37 already identified BLBC (CK5 and/or EGFR+) and the percentage of basal-like tumors in the pool of TN cases of our series rounds the 65% (71/109). This rate is similar to the one identified by Bertucci (Bertucci et al., 2008), where 70% of IHQ TN tumors presented a basal phenotype by GEP. It is worth noticing that using P-cadherin, CK14 and Vimentin to recruit BLBC from the pool of tumors that could not be classified using only CK5 and EGFR as basal makers, these newly identified BLBC are clinically similar to basal-like tumors identified by Nielsen's criterion, since the majority of the cases presented high histological grade and there are no significant differences in what concerns overall survival of the patients.

Although CK5 and EGFR have been consistently used to recognize BLBC, P-cadherin, CK14 and

Vimentin could also be recruited for an immunohistochemical recognition of BLBC (Paredes et al., 2002, 2007a,b; Matos et al., 2005; Livasy et al., 2006; Rodriguez-Pinilla et al., 2007). Our results showed that these three markers can reliably identify the basal phenotype, especially when associated to CK5, and can be alternative options in this setting. We also demonstrate that P-cadherin, due to its high sensitivity, can recognize possible BLBC among the IHC TN tumors, probably identifying patients with poor prognosis that can benefit from this differential recognition. Pathologists have faced continuous changes in the diagnostic approach of breast cancer and, regarding its classification, it is still controversial whether or not the histological classification should be replaced by the "molecular" taxonomy. Therefore, it is essential to move towards a standardized methodology to establish an IHC panel of biomarkers to the most appropriate recognition of basal-like breast carcinomas.

Conflict of interest. The authors declare that they have no conflict of interest.

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Publications

Paper VII

Cytological Criteria to Predict Basal Phenotype of Breast Carcinomas

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Breast carcinoma is a heterogeneous disease. It can be classified into phenotypes based on the expression of certain proteins, with distinct differences in prognosis. The basal phenotype is associated with worse prognosis and it still remains without specific treatment. However, there is currently no international consensus on the cytological criteria that could predict this phenotype. The purpose of the study was to evaluate the cytological criteria in fine-needle aspiration biopsy and to identify their association with the basal phenotype of breast carcinoma. Fine-needle aspiration biopsy specimens and tissue sections (mastectomy specimen) from 74 cases of high-grade invasive ductal breast carcinomas were consecutively retrieved from the files of three institutions. Breast carcinomas were studied using the tissue microarray technique, being classified into phenotypes: luminal A, luminal B, HER2 overexpression, and basal. The cytological criteria for all cases were reviewed blindly by two pathologists according to five cytological criteria: cellularity, cell pattern, presence of necrosis, nucleoli, and nuclear atypia. Exact Fisher test was used to test the association between cytological criteria and the phenotypes of breast carcinoma. Necrosis was present in 64.7% of basal breast carcinomas, and 31.1% of nonbasal breast carcinomas, and that result was statistically significant, showing an odds ratio (OR) of 3.80. The basal phenotype, compared with the luminal A, showed more necrosis (OR = 6.97), present/prominent nucleoli (OR = 8.18), and cellularity more frequently (OR = 18.03). Necrosis, as well as present/prominent nucleoli and abundant cellularity are criteria more frequently associated to the basal phenotype of breast carcinoma. Diagn. Cytopathol. 2009;37:809–814.

Key Words: breast cancer; fine needle aspiration cytology; basal cell cancer; cytology

Since therapeutic planning is frequently made as a preoperative multidisciplinary triple approach and fine-needle aspiration cytology (FNAC) is an integral part of this, it is important to gather as much prognostic information from the cytological specimen as possible.[1–7] This procedure has become widely accepted as a first-line diagnostic procedure for breast lesions and as a reliable diagnostic tool with both high sensitivity and specificity with minimum complications.[8–12] Emerging data demonstrate that stratification of tumors by gene-expression profiles divides breast carcinoma into a mixture of at least two main types, according to hormone estrogen receptor (ER) expression. The hormone receptor-negative group has two subtypes: human epithelial receptor 2 (HER2) overexpressing and basal-like. The hormone receptor-positive group has two subtypes: luminal A and luminal B.[13–15] Basal breast carcinomas represent one of the most intriguing subtypes because there is no efficient therapy against these lesions, which are often associated with poor prognosis.[16–18]

Basal breast carcinomas are thought to arise from the basal epithelial layer of the breast duct. This subgroup has morphology characteristics consisting of a high proliferate rate, central necrosis, and pushing border.[6,17,19]

FNAC offers a suitable alternative to biopsy in a variety of clinical settings, in which it may be useful to obtain material to study diagnostic, prognostic, and predictive markers. The progress of "specific" therapies based on antibody response will certainly obligate the cytologists to actively participate in the decision-making for therapeutic options for patients.[7,20]

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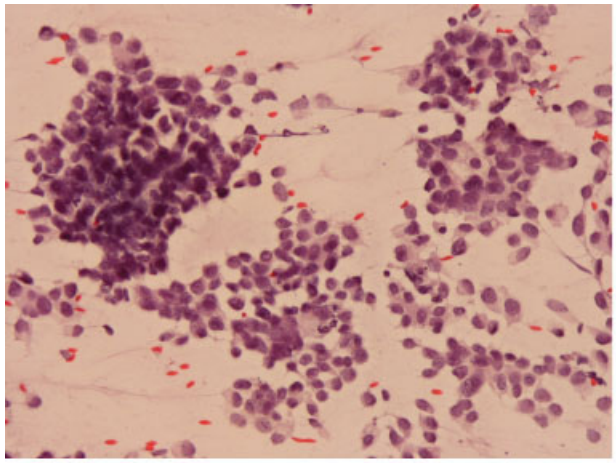

Fig. C-1

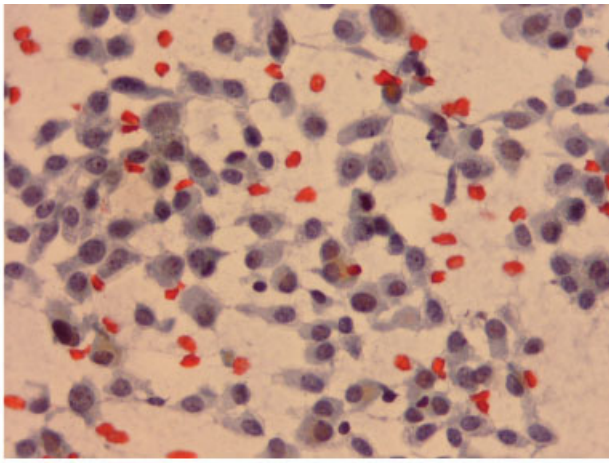

Fig. C-2

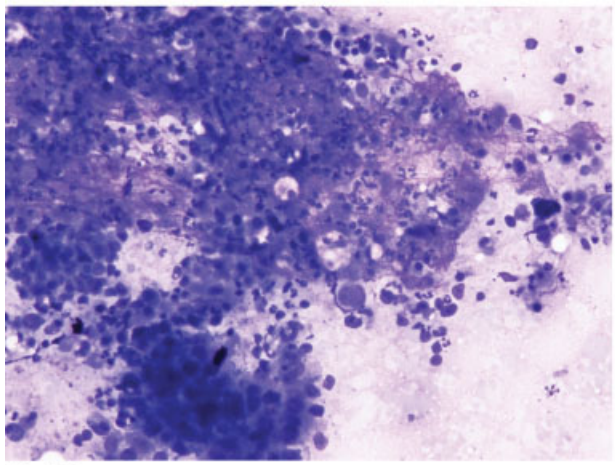

Fig. C-3

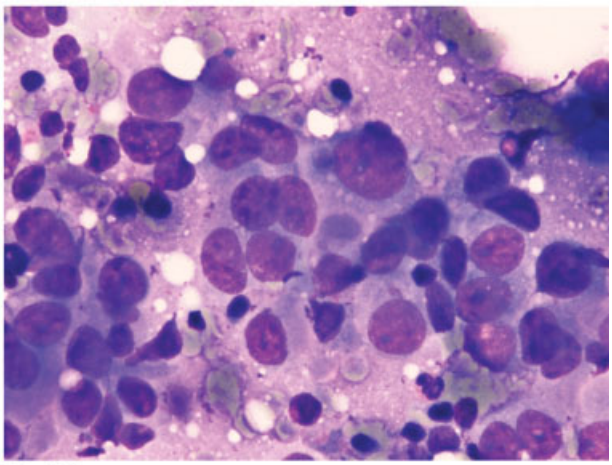

Fig. C-4

Figs. C-1–C-4. Fig. C-1. Abundant cellularity (Papanicolaou stain, ×200). **Fig. C-2.** Dissociated cell pattern (Papanicolaou stain, ×400). **Fig. C-3.** Necrosis (May-Grünwald Giemsa stain, ×400). **Fig. C-4.** Severe nuclear atypia (May-Grünwald Giemsa stain, ×400).

To date, the basal subgroup has been defined by gene arrays analysis. However, this method is very expensive. The purpose of this study was to determine whether some cytological criteria could predict the basal phenotype of breast carcinoma, using as gold standard a series of breast carcinomas classified according to the current molecular classification by means of tissue microarray (TMA) technique.

Methods

Breast Carcinoma Samples

FNAC specimens and tissue sections (mastectomy specimen) from 74 cases of histological grade III invasive ductal breast carcinomas, diagnosed between 2000 and 2007, were identified from the patient database at the department of Pathology, Federal University of Santa Catarina, Florianopolis, Brazil, IMP Medical Laboratory, Florianopolis, Brazil, and at the Hospital Sao Joao, University of Porto, Portugal. Formalin-fixed, paraffin-embedded tissues, and FNAC specimens of 74 grade III invasive ductal breast carcinomas were consecutively retrieved from the files of these institutes. They were examined using tissue microarray (TMA) technology and immunohistochemistry. The use of these specimens and data for research purposes was approved by the Ethics Committee of the Federal University of Santa Catarina, Florianopolis, Brazil.

Cytological Criteria

All FNAC specimens were previously stained with Papanicolaou stain and May-Grünwald Giemsa stain. The cytological parameters for all cases were reviewed by two pathologists (F.S. and R.M.D.), through the review of the FNAC specimens in the multihead microscope in order to identify the presence of five individual cytological criteria: cellularity, nuclear atypia, cell pattern, nucleoli, and presence of necrosis.[20] The Figures C-1–C-4 illustrate some of the criteria analyzed in this study.

Table I. Sources and Dilutions of Primary Antibodies Used in This Immunohistochemistry Study

Antibody	*Clone*	*Manufacturer*	*Dilution*	*Time of incubation (minutes)*	*Antigen retrieval (minutes)*
CK5	XM26	LabVision	1:50	60	30
P-cadherin	Clone 56	LabVision	1:50	60	30
EGFR	31G7	Zymed	1:100	60	30
ER	SP1	LabVision	1:150	30	30
HER2	SP3	LabVision	1:80	30	30

Tissue Microarray Construction

Representative areas of the invasive breast carcinomas were carefully selected on hematoxylin and eosin (H&E) stained sections and marked on individual paraffin blocks by a pathologist (R.M.D.). Two tissue cores (2 mm in diameter) were obtained from each selected specimen and precisely deposited into a recipient paraffin block using a TMA workstation (TMA builder ab1802, Abcam, Cambridge, UK). Four TMA blocks were constructed, each containing 24 tissue cores, and arranged in a 4 × 6 sector. In each TMA block, non-neoplastic breast and testicular tissue cores were also included as controls and TMA guide, respectively. After construction, 2-μm tissue sections were cut and adhered to Super frost plus glass slides. An H&E stained 2-μm section from each block was reviewed to confirm the presence of morphological representative areas of the original lesions. All markers were assayed in TMAs.

Immunohistochemistry

The molecular phenotype was established in the paraffin block, with the use of specific immunohistochemical markers, through the TMA technique. Like Nielsen et al.[21] we also classified each tumor in a practical way based on its ER and HER2 expression. A total of 74 cases were immunohistochemically interpretable to allow sample characterization into one of five groups. If a tumor was ER-positive, it would be classified as luminal. If a tumor was ER-positive and HER2-negative (0, 1, or 2+), it would be classified as luminal A. However, if it was ER-positive and HER2-positive, it would be classified as luminal B. If a tumor was ER-negative and HER2-positive, it would be classified as HER2-overexpressing, and if it was both ER- and HER2-negative but positive for at least one basal marker (P-cadherin and/or EGRF and/or CK5), it would be classified as basal. If a tumor did not show expression for any of these markers, it would be classified as null phenotype and would not be considered in the remaining analyses.

Immunohistochemical staining for ER, HER2, and CK5 was performed using the streptavidin-biotin-peroxidase technique (Laboratory Vision Corporation, Fremont, CA) in each set of four glass slides comprising the TMAs, whereas P-CAD and EGFR used the HRP-labeled polymer (DakoCytomation, Carpinteria, CA). The antigen retrieval times, clone antibodies, dilutions, and suppliers are listed in Table I. Positive and negative controls were included in each run in order to guarantee the reliability of the assays. Non neoplastic breast tissue cores, as well as normal breast surrounding the neoplastic cells, were considered internal controls for most of the antibodies tested: CK5, P-cadherin, EGFR (myoepithelial cells); ERα, (epithelial cells). All slides were analyzed by two pathologists (F.S. and R.M.D.) in a multihead microscope (Leica MDL, Germany).

Statistical Analysis

The data were described in absolute frequencies (n) and relative frequencies (%) to evaluate the association of cytological criteria. These cytological criteria were compared between groups of breast carcinomas by means of the Fisher exact test, with a confidence interval of 95%. P values of <0.05 (two-tailed) were considered as statistically significant. Statistical analyses were performed with SAS statistical software, Version 9.02.

Results

A total of 74 cases were immunohistochemically interpretable to allow sample characterization into one of five groups. We observed that basal type comprised 23.0% of all tumors, whereas luminal A and luminal B comprised 35.1% and 5.4%, respectively. HER2-overexpressing tumors represented 20.3% of the series, and null phenotype, 16.2% (Table II).

Table III shows cytological criteria correlated with molecular profile. Necrosis was present in 64.7% of breast carcinomas with basal phenotype, and it was present in 31.1% of nonbasal (luminal A, luminal B, and HER2-overexpressing) carcinomas, and this result was statistically significant, showing OR of 3.80 ($P = 0.0404$) (Tables III and IV).

Other cytological criteria to identify the basal phenotype were higher cellularity (76.5% of cases), intense atypia (76.5% of cases), mild or moderate cohesion cellular in 64.7% of cases, and nucleoli was present in 88.2% of cases. The frequency of these criteria, however, did not

Table II. Frequencies of Immunohistochemically Defined Phenotypes of Breast Carcinomas in 74 Informative Tumors for the Tested Markers Using TMA

Phenotype of breast carcinoma	*ER*	*HER2*	*P-cadherin and/or EGFR and/or CK5*	*Frequency n (%)*
Luminal A	Positive	Negative	Positive/negative	26 (35.1)
Luminal B	Positive	Positive	Positive/negative	4 (5.4)
Basal	Negative	Negative	Positive	17 (23.0)
HER2-overexpressing	Negative	Positive	Positive/negative	15 (20.3)
Null[a]	Negative	Negative	Negative	12 (16.2)

[a]If a tumor did not show expression for any of immunohistochemical markers, it would be classified as null phenotype and would not be considered in the remaining analyses.

Table III. Distribution of the Identified Individual Cytological Criteria in the Examination of the Fine-Needle Aspiration Biopsy With the Diagnosis of Molecular Phenotype of the Breast Carcinoma

	Basal		*Luminal A*		*Luminal B*		*HER2-overexpressing*		*Nonbasal*[b]	
Cytological criteria	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Cellularity										
Scant	0	0.0	8	33.3	1	25.0	2	13.3	11	25.6
Moderate	4	23.5	5	20.8	0	0.0	5	33.3	10	23.3
Abundant	13	76.5	11	45.8	3	75.0	8	53.3	22	51.2
Indeterminate/excluded[a]	0		2		0		0		2	
Cell pattern										
Dissociated	6	35.3	4	16.7	1	25.0	2	13.3	7	16.3
Fairly equal representation of clustered and dissociated cells	11	64.7	12	50.0	3	75.0	10	66.7	25	58.1
Clustered	0	0.0	8	33.3	0	0.0	3	20.0	11	25.6
Indeterminate/excluded[a]	0		2		0		0		2	
Necrosis										
Present	11	64.7	5	20.8	3	75.0	6	40.0	14	31.1
Not present	6	35.3	19	79.2	1	25.0	9	60.0	29	67.4
Indeterminate/excluded[a]	0		2		0		0		2	
Nucleoli										
Inconspicuous	2	11.8	12	52.2	1	25.0	0	0.0	13	32.5
Present	3	17.6	11	47.8	2	50.0	3	23.1	16	40.0
Present and prominent	12	70.6	0	0.0	1	25.0	10	76.9	11	27.5
Indeterminate/excluded[a]	0		3		0		2		5	
Nuclear atypia										
Mild	0	0.0	6	25.0	0	0.0	0	0.0	6	14.0
Moderate	4	23.5	15	62.5	2	50.0	4	26.7	21	48.8
Severe	13	76.5	3	12.5	2	50.0	11	73.3	16	37.2
Indeterminate/excluded[a]	0		2		0		0		2	
Total of cases	17	27.4	26	41.9	4	6.5	15	24.2	45	72.6

[a]It was considered as indeterminate cytological criteria when it was not possible to characterize in the examination of fine-needle aspiration biopsy specimen. Those cases were excluded from percentage analyses.
[b]Luminal A, luminal B, and HER2-overexpressing.

present a significant difference compared with frequency observed in other phenotypes of breast carcinoma (Tables III and IV).

Referring to the luminal A phenotype, 33.3% of the FNAC specimens showed scant celullarity, 25.0% showed mild atypia, 52.2% showed inconspicuous nucleoli, and only 20.8% showed necrosis (Table IV). The basal subtype, compared with the luminal subtype, showed more necrosis (OR = 6.97, CI 95%: 1.72–28.25), and prominent nucleoli (OR = 8.18, CI 95%: 1.51–44.21). The cytological criteria cellularity showed bordering significance to differentiate the basal phenotype from the luminal A, showing an odds ratio (OR) of 18.03 (CI 95%: 0.96–337.81).

Discussion

Clinicians can reduce the risk of missed diagnoses of breast carcinomas to 1% by using the triple test approach, which is based on the correlation of clinical information, imaging, and cytological diagnosis of FNAC to direct patient management. Since therapeutic planning can be made preoperatively on the basis of the cytological report, it is important to gather as much prognostic information from the cytological specimen as possible.[20] FNAC is recommended as a first-line procedure given the ease of performing the technique, the rapid turnaround time to obtain a diagnosis, and the low cost compared with biopsy or surgery as an initial approach.[11]

Table IV. Correlation Between Cytological Criteria Comparing the Basal Phenotype and Other Phenotypes of Breast Carcinoma-Defined Immunohistochemically

Cytological criteria	*P*[a]	*OR (CI 95%)*
Cellularity (scant vs. moderate/abundant)	0.0257	
Basal × HER2-overexpressing		6.48 (0.29–146.54)
Basal × luminal A		18.03 (0.96–337.81)
Basal × luminal B		15.00 (0.50–449.55)
Basal × nonbasal[b]	0.0247	12.38 (0.69–222.95)
Cell pattern (dissociated vs. fairly equal representation of clustered and dissociated cells /clustered)	0.3968	
Basal × HER2-overexpressing		0.28 (0.05–1.69)
Basal × luminal A		0.37 (0.08–1.58)
Basal × luminal B		0.76 (0.09–6.45)
Basal × nonbasal[b]	0.1628	0.36 (0.10–1.29)
Necrosis (present)	0.0151	
Basal × HER2-overexpressing		2.75 (0.66–11.54)
Basal × luminal A		6.97 (1.72–28.25)
Basal × luminal B		0.61 (0.05–7.24)
Basal × nonbasal[b]	0.0404	3.80 (1.16–12.38)
Nucleoli (inconspicuous vs. present/present and prominent)	0.0013	
Basal × HER2-overexpressing		0.23 (0.01–5.22)
Basal × luminal A		8.18 (1.51–44.21)
Basal × luminal B		2.50 (0.17–37.26)
Basal × nonbasal[b]	0.1871	3.61 (0.72–18.19)
Nuclear atypia (mild vs. moderate/severe)	0.0236	
Basal × HER2-overexpressing		1.13 (0.02–60.37)
Basal × luminal A		12.30 (0.64–234.91)
Basal × luminal B		3.89 (0.07–224.24)
Basal × nonbasal[b]	0.1703	6.07 (0.32–113.83)

[a]Fisher exact test.
[b]Luminal A, luminal B, and HER2-overexpressing.
OR = odds ratio; CI 95% = confidence interval of 95%.

In the present study we found that the presence of necrosis in the cytological smears is one of the most important cytological criteria in distinguishing basal and nonbasal breast carcinomas. Recently, Chivukula et al. found similar results on core needle biopsies of the breast.[22] In fact, since the seminal paper of Tsuda et al.[23] that described the presence of large, central areas of necrosis as one of the most important morphological criteria of carcinomas with basal-like differentiation, other articles having been confirming these findings.[6,17,19,24,25] However, this is the first study that demonstrates the presence of necrosis as important criteria on the characterization of basal-like breast carcinomas in FNAC. Another result from our study is that basal-like breast carcinomas presented more frequently abundant cellularity, necrosis, and presence/prominent nucleoli if compared with the subtype luminal A. However, it is important to highlight that the criteria cellularity presented borderline significance, with an OR of 18.03 ($P = 0.0257$; CI 95%: 0.96–337.81). This fact can have occurred due to the insufficiency of the analyzed sample in demonstrating the difference, which happens when the event is rarer or when few differences are present between the data. In the analysis of the cellularity of the basal and luminal phenotypes, the only category with a clear difference was scant cellularity, with eight cases presenting scant cellularity in luminal phenotype and no cases in basal phenotype. The results found in this study warrant special attention because the basal breast carcinomas have very distinct therapeutic forms in relation to the luminal carcinoma.

Basal breast carcinomas have been reported to constitute 17–23% of sporadic grade III invasive ductal carcinomas.[5,6,26–29] These data are according to this study: we found 23% of invasive grade III breast carcinomas presenting the basal phenotype.

It has been shown in studies carried out on behalf of the Breast Cancer Linkage Consortium that a combination of morphology, negativity for ER and "basal" keratin positivity could provide a powerful predictor tool for *BRCA1* mutation status and hence may be useful in selecting patients for *BRCA1* mutation testing.[17] Many women with mutations in the *BRCA1* gene do not have a family history of breast and/or ovarian cancer. Therefore, detecting a cancer in a young woman with cytological criteria and immunophenotype indicating a basal tumor can help alert doctors to the possibility of a familial predisposition.

We are aware of the limitations of the present study due to the reduced number of high-grade invasive ductal breast carcinoma cases available for analysis and, therefore, more studies concerning this subject are necessary using a larger sample of analyzed cases. However, the cytological criteria found associated with negativity for ER and HER2, and a positivity for basal immunohistochemical markers such as CK5, P-cadherin, and EGFR, could assist in the confirmation of diagnosis of breast cancer with basal phenotype. Due to the awareness of the aggressive nature of this subtype of tumor, it is of great clinical interest to establish its diagnosis as early as possible. Therefore, in the presence of cytological findings of necrosis, present/prominent nucleoli, and abundant cellularity, we recommend investigating the possibility of dealing with a basal breast carcinomas and, if possible, trying to confirm this diagnosis through the immunohistochemical analysis.

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