



INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

PROGNOSTIC VALUE OF METHYLATION MARKERS IN BREAST CANCER

ANA TERESA PINTO TEIXEIRA MARTINS

Porto, 2009

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**DISSERTAÇÃO DE MESTRADO EM ONCOLOGIA
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TABLE OF CONTENTS

Abbreviations	9
Summary	13
Resumo	17
Résumé	21
Introduction	25
I. Breast cancer	27
1. Epidemiology	27
2. Risk Factors	29
3. Etiology	32
4. Diagnosis and Grading	34
5. Staging and Prognosis Indicators	38
II. The molecular biology of breast cancer	41
1. Genetics	41
2. Epigenetics	42
III. Genes	49
1. CCND2	49
2. RASSF1A	51
3. APC	53
Objectives	55
Materials and Methods	59
I. Patients	61
II. Cytological Preparations	61
III. DNA extraction	61
IV. Methylation Analysis	62
1. Bissulfite Modification	62
2. QMSP Analysis	63
V. Statistical Analysis	64
Results	67
Discussion	77
Conclusions	83
Acknowledgments	87
References	91

‘Nunca mais
Caminharás nos caminhos naturais.
Nunca mais te poderás sentir
Invulnerável, real e densa -
Para sempre está perdido
O que mais do que tudo procuraste
A plenitude de cada presença.

E será sempre o mesmo sonho, a mesma ausência’

Sophia de Mello Breyner Andresen

To my grandmother

ABBREVIATIONS

ADN – Ácido desoxirribonucleic
 APC – Adenomatous polypolis coli
 AUC – Area under the curve
 Bcl2 - B cell lymphoma 2
 BRCA1 – Breast cancer 1
 BRCA2 – Breast cancer 2
 BAAF – Biopsia aspirativa por agulha fina
 CCND2 - Cyclin D2
 CDH1 – E-cadherin
 CDK - Cyclin-dependent kinases
 CISH - Chromogenic in situ hybridization
 DAPK1 - Death-associated protein kinase 1
 DNA – Desoxirribonucleic Acid
 DNMTs - DNA methyltransferases
 EDTA – Ethylenediamine teracetic acid
 ER - Estrogen receptor
 EtOH – Ethanol
 FISH - Fluorescent in situ hybridization
 FNA – Fine needle aspiration
 GSTP1 – Glutathione s-transferase pi 1
 H&E – Hematoxylin & Eosin
 HER2 - Human epidermal growth factor receptor 2
 HIN-1 – High in normal 1
 hMLH1 - MutL homolog 1
 HRP – Hormone Replacement Therapy
 IGF2 – Insulin-like growth factor 2
 M – Molar
 mg - Milligram
 MGMT – O(6)-methylguanine-DNA-methytransferase
 mL - Milliliter

mM - Millimolar
 NaOH – Sodium hidroxide
 NaCl – Sodium chlorine
 nM – Nanomolar
 NSCLC – Non-small cell lung carcinoma
 OC – Oral contraceptives
 PBS – Phosphate saline buffer
 PCR – Polymerase chain reaction
 PgR - Progesterone receptor
 PK – Proteinase K
 PTEN - Phosphatase and tensin homolog
 QMSP – Quantitative methylation-specific PCR
 RAR β - Retinoic acid receptor β
 RASSF1A - Ras association domain family 1
 RB – Retinoblastoma
 ROC – Receiver Op
 rpm – Rotations per minute
 RR – Relative Risk
 SCLC – Small cell lung carcinoma
 SDS - Sodium dodecyl sulfata
 SE – Buffer solution
 TGS – Tumor supressor gene
 THBS1 – Thrombospondin 1
 TNM – Tumor-Node-Metastasis
 TP53 - Tumor protein p53
 TWIST – Twist homolog
 UTR – untranslated regions
 Wnt – Wingless-type oncogene
 μ L – Micro liter

SUMMARY

Breast cancer is a major cause of cancer-related morbidity and mortality in developed countries. Fine needle aspiration biopsy (FNA) of suspicious breast lesions provides a relatively simple, minimally invasive and rapid mean of triaging patients to more complex diagnostic procedures. Previously, we reported on the feasibility of detecting aberrant gene promoter methylation (an epigenetic alteration commonly affecting cancer-related genes) in FNA washings and demonstrated that the accuracy of cytological diagnosis could be augmented using a quantitative methodology for the assessment of DNA methylation. Herein, we aimed at the confirmation of the diagnostic performance of methylation markers and also at the evaluation of the prognostic value of quantitative promoter methylation at three gene loci (*APC*, *CCND2*, and *RASSF1A*) in a large series of FNA washings from breast lesions.

The methylation levels of the three gene promoters were assessed by quantitative methylation-specific PCR in bisulfite-modified DNA from 211 FNA washings, comprising 178 carcinomas and 33 benign lesions, histopathologically confirmed. Receiver operator characteristic (ROC) curve analysis was used to determine the diagnostic performance of the gene panel in distinguishing cancer from non-cancerous lesions. Relevant clinicopathologic data (age, tumor grade, pathologic stage, and hormone receptor status) and time to progression and/or death from breast cancer were correlated with methylation findings. Log-rank test and Cox regression model were used to identify which epigenetic markers were independent predictors of prognosis.

APC, *CCND2*, and *RASSF1A* methylation levels differed significantly between malignant and benign lesions. ROC curve analysis confirmed the diagnostic performance of the gene panel. An optimal balance between sensitivity and specificity (approximately 80% for both) was achieved when positivity for two markers was defined as the criteria to identify malignant lesions. At a median follow-up of 57.7 months, 19 (10.7%) patients died from breast cancer and 32 (18.0%) patients had recurrent disease. In univariate analysis, stage was significantly associated with overall, disease-specific and disease-free survival, whereas tumor grade was associated with disease-specific and disease-free survival. Remarkably, hypermethylation of *RASSF1A* was significantly associated with worse disease-free survival. In the final multivariate analysis, pathologic stage, tumor grade and high-methylation of *RASSF1A* were significantly and independently associated with unfavourable prognosis.

This study confirms that quantitative gene promoter methylation augments the diagnostic performance of cytopathology, providing a helpful ancillary tool to cytomorphological evaluation. Importantly, and in addition to standard clinicopathologic parameters, *RASSF1A* high-methylation level was shown to be an independent predictor of worse outcome in breast cancer. Further studies addressing the development of predictive models for pre-operative staging and therapy response based on epigenetic biomarkers might also provide valuable tools for breast cancer patient management.

RESUMO

O cancro da mama é a principal causa de morbilidade e mortalidade relacionada com cancro nos países desenvolvidos. A biopsia aspirativa por agulha fina (BAAF) de lesões mamárias suspeitas fornece um meio relativamente simples, minimamente invasivo e de rápida triagem de pacientes, antes de serem submetidos a procedimentos diagnósticos mais complexos. Em trabalhos prévios nós reportamos a viabilidade da detecção de metilação aberrante do promotor do gene (uma alteração epigenética que normalmente afecta genes relacionados com o cancro) na lavagem de agulha de BAAF e demonstramos que o rigor do diagnóstico citológico pode ser aumentado utilizando uma metodologia quantitativa para a avaliação da metilação do ADN. Neste trabalho, visamos a confirmação do desempenho diagnóstico dos marcadores de metilação e também a avaliação do valor prognóstico da metilação quantitativa do promotor em três *loci* (gene *APC*, *CCND2* e *RASSF1A*) numa longa série de lavagens de agulha de BAAF de lesões da mama.

Os níveis de metilação dos três promotores dos genes foram avaliados por PCR quantitativo específico para metilação em ADN modificado pelo bissulfito em 211 lavagens de BAAF, compreendendo 178 carcinomas e 33 lesões benignas, confirmadas histopatologicamente. A curva ROC (Receiver operator characteristic) foi utilizada para determinar o desempenho diagnóstico do painel de genes quanto à distinção entre cancro e lesões não-cancerosas. Os dados clínico-patológicos relevantes (idade, grau tumoral, estadio patológico e *status* dos receptores hormonais) e o tempo de progressão e/ou morte por cancro da mama foram correlacionadas com os resultados da metilação. O teste *log-rank* e regressão *Cox* foram utilizados para identificar que marcadores epigenéticos foram considerados como factores independentes de prognóstico.

Os níveis de metilação do *APC*, *CCND2* e *RASSF1A* diferiram significativamente entre lesões benignas e malignas. A análise da curva ROC confirmou o desempenho diagnóstico do painel de genes. Um equilíbrio entre a sensibilidade e especificidade (cerca de 80% para ambos) foi alcançado quando a positividade para dois marcadores foi definida como critério para identificar lesões malignas. Num *follow-up* médio de 57,7 meses, 19 (10,7%) pacientes morreram de cancro da mama e 32 (18,0%) pacientes tiveram recorrência da doença. Na análise univariada, o estadio foi associado significativamente com a sobrevivência global, sobrevivência específica de doença e sobrevivência livre de doença, enquanto que o grau do tumor foi apenas associado com sobrevivência

específica de doença e sobrevivência livre de doença. Notavelmente, a hipermetilação do *RASSF1A* foi significativamente associada com pior sobrevida livre de doença. Na análise final multivariada, o estadiamento patológico do tumor e alto grau de metilação do *RASSF1A* foram significativamente e independentemente associados com um prognóstico desfavorável.

Este estudo confirma que a metilação quantitativa do promotor do gene aumenta o desempenho diagnóstico da citopatologia, fornecendo uma ferramenta útil para auxiliar a avaliação citomorfológica. Mais importante ainda, e para além dos parâmetros clínico-patológicos, o alto nível de metilação do *RASSF1A* mostrou ser preditor independente de pior prognóstico no cancro da mama. Novos estudos abordando o desenvolvimento de modelos preditivos para o estadiamento pré-operatório e a resposta à terapêutica baseada em marcadores epigenéticos poderiam ser úteis na tentativa de fornecer ferramentas valiosas para a gestão de pacientes com cancro de mama.

RÉSUMÉ

Le cancer du sein est une cause majeure de cancer liés à la morbidité et la mortalité dans les pays développés. Fine needle aspiration biopsy (FNA) de lésions mammaires suspectes offre un moyen relativement simple, peu invasive et rapide de trier les patients à des procédures de diagnostic plus complexes. Auparavant, nous avons signalé sur la faisabilité de détecter la méthylation aberrante des gènes promoteur (une modification épigénétique qui influencent généralement gènes liés au cancer) dans les lavages FNA et montré que la précision du diagnostic cytologique peut être augmentée en utilisant une méthode quantitative pour l'évaluation de la méthylation de ADN. Présentes, nous visant à la confirmation de la performance diagnostique des marqueurs de méthylation et également à la évaluation de la valeur pronostique de la méthylation du promoteur quantitative à trois loci (*APC*, *CCND2*, et *RASSF1A*) dans une grande série de lavages FNA de lésions du sein.

Les niveaux de méthylation des trois promoteurs de gènes ont été évalués par PCR quantitative spécifique de méthylation dans bisulfite modifié ADN à partir de 211 lavages FNA, comprenant 178 carcinomes et 33 lésions bénignes, histopathologique confirmée. La fonction d'efficacité du récepteur (ROC) analyse de la courbe a été utilisée pour déterminer les performances diagnostiques du panel de gènes dans le cancer de distinguer les lésions non cancéreuses. Clinicopathologic données pertinentes (âge, grade de la tumeur, le stade pathologique, et le statut des récepteurs hormonaux) et le temps jusqu'à progression et / ou la mort d'un cancer du sein ont été corrélés avec les résultats de méthylation. Test du log-rank et un modèle de Cox ont été utilisés pour identifier les marqueurs épigénétiques aient été des facteurs prédictifs indépendants de pronostic.

Les niveaux de méthylation du *APC*, *CCND2* et *RASSF1A* différait sensiblement entre les lésions malignes et bénignes. Analyse de la courbe ROC a confirmé les performances diagnostiques du panel de gènes. Un équilibre optimal entre la sensibilité et de spécificité (environ 80% pour les deux) a été atteint lorsque la positivité de deux marqueurs a été défini que les critères à identité lésions malignes. Lors d'un suivi médian de 57, 7 mois, 19 (10, 7%) patients sont décédés d'un cancer du sein et 32 (18,0%) patients avaient une maladie récurrente. En analyse univariée, le stade était significativement associée à l'ensemble, des maladies particulières et la survie sans maladie, alors que le grade tumoral a été associée à des maladies particulières et la survie sans

maladie. Fait remarquable, hyperméthylation de RASSF1A était significativement associée à une dégradation de la survie sans maladie. En analyse multivariée finale, stade pathologique, grade de la tumeur et la méthylation élevée de RASSF1A étaient significativement et indépendamment associée à un pronostic défavorable.

Cette étude confirme que la méthylation quantitative du gène promoteur augmente la performance diagnostique de la cytopathologie, fournissant un outil accessoire utile à l'évaluation cytomorphologique. Fait important, et en plus des paramètres standard clinicopathologic, RASSF1A haut niveau de méthylation a été révélée être un facteur prédictif indépendant de mauvais résultats dans le cancer du sein. D'autres études portant sur le développement de modèles prédictifs pour la pré-mise en scène du dispositif et l'intervention de thérapie basée sur les marqueurs biologiques épigénétiques pourraient aussi fournir de précieux outils pour la gestion du cancer du sein.

INTRODUCTION

I. BREAST CANCER

1. EPIDEMIOLOGY

Among all female cancers diagnosed worldwide in 2002, breast cancer accounted for 23%, being the most common cancer in women, with an estimated 1.15 million new cases that year. More than half of all cases occurred in industrialized countries, with 361,000 diagnosed in Europe (27.3% of cancers in women) (Figure 1). Partially, the high incidence in the most affluent world areas is likely due to the presence of screening programs that detect early invasive cancers, some of which would otherwise have been diagnosed later or not at all (Globocan, 2002; Parkin and Fernandez, 2006).

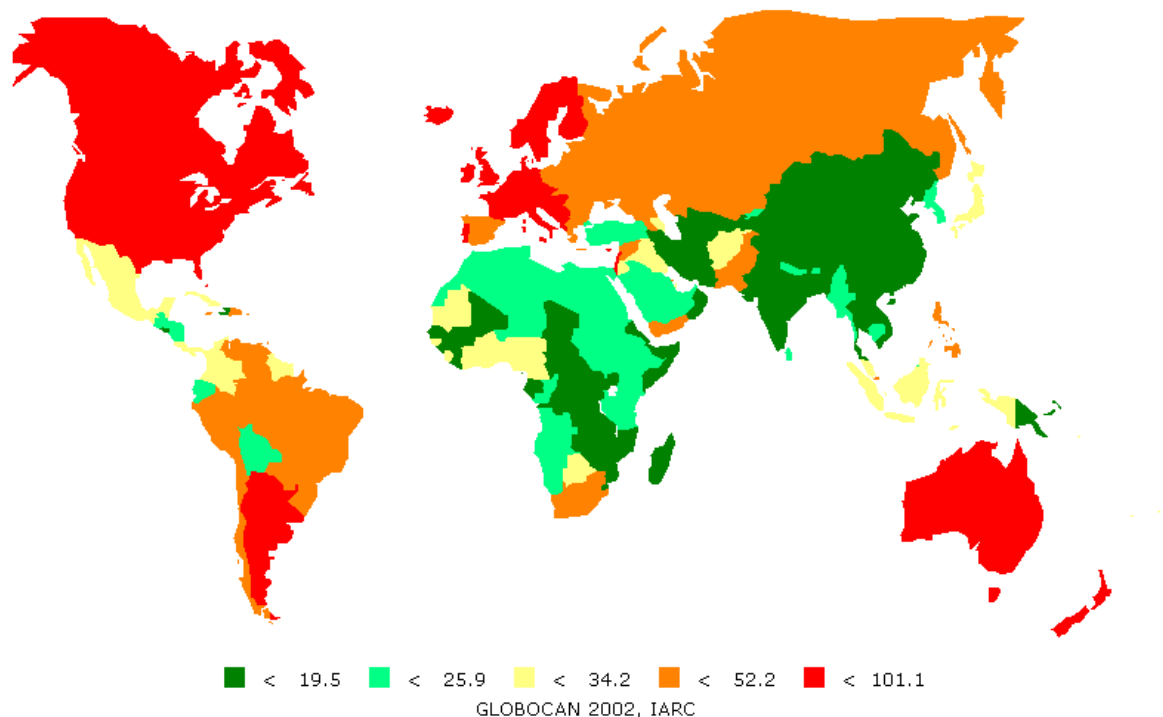


Figure 1: Breast cancer incidence rates worldwide, age-standardized (world standard) rates (per 100,000) - Globocan 2002

Because breast cancer prognosis is generally fair, it ranks as the fifth cause of cancer-related deaths, although it remains the leading cause of cancer mortality in women (the 411,000 annual deaths represent 14% of female cancer deaths) (Figure 2).

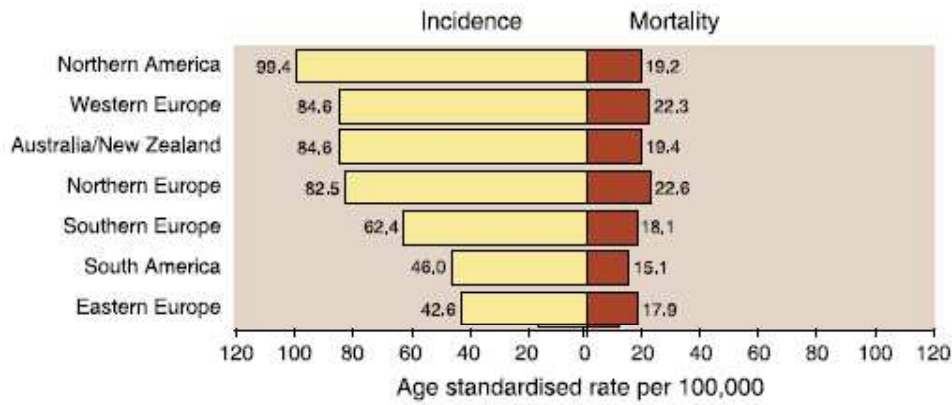


Figure 2: Breast cancer incidence and mortality rates per 100000 by region or country (Adapted from Parkin and Fernandez, 2006)

In Portugal in 2002 (Figure 3), 4309 new cases were diagnosed, representing 26% of all cancers in women, and of all cancer deaths in women, breast cancer was responsible for 17.4% (Globocan, 2002).

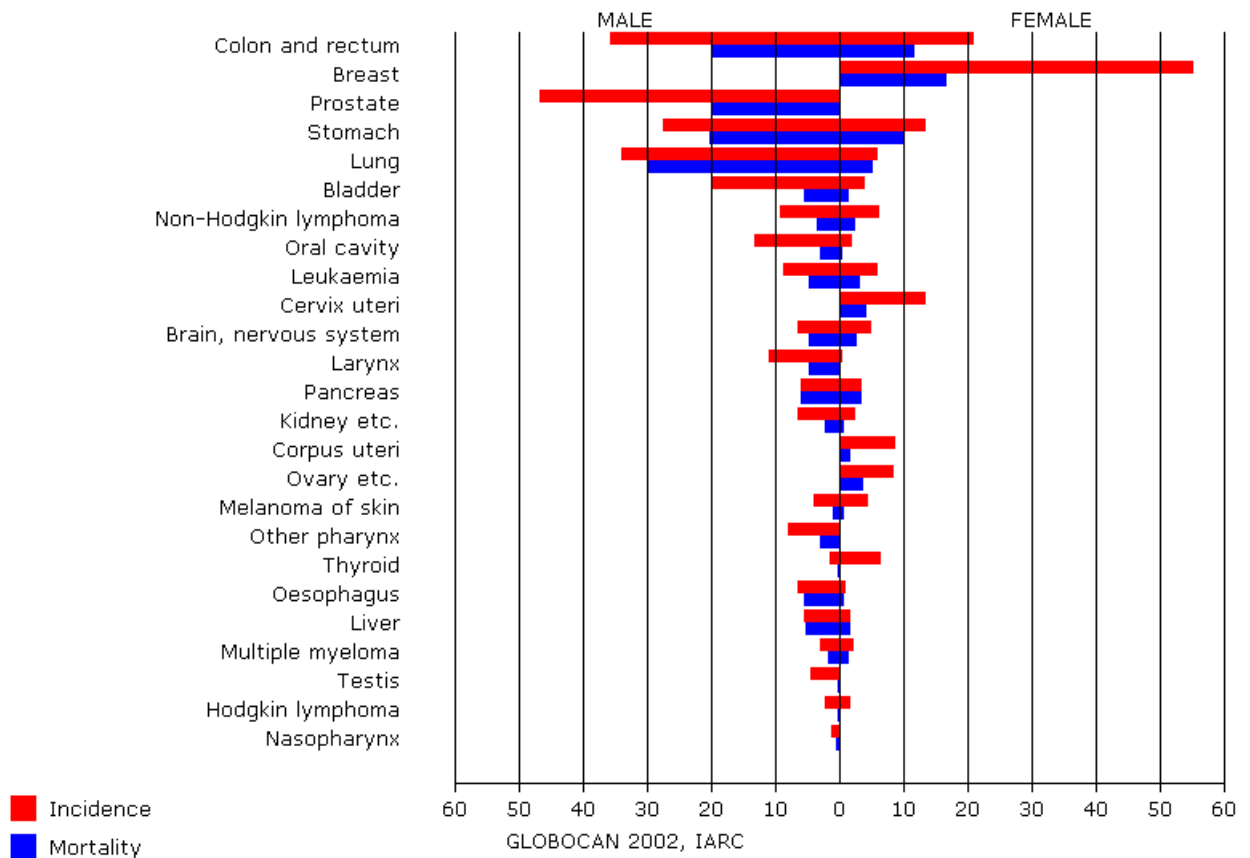


Figure 3: Cancer incidence rates, females, all ages, Portugal - Globocan 2002

2. RISK FACTORS

Breast cancer is a complex disease that results from the interaction of multiple environmental, hormonal, and lifestyle risk factors, as well as the individual's genomic profile. Although inherited risk factors are not modifiable, most lifestyle factors can be altered, leading to opportunities for breast cancer risk reduction for many women (Pruthi et al., 2007).

Although a risk factor is defined as a characteristic of individual patients that increase their chance of developing breast cancer when compared to the risk of the general population, the absence of these risk factors does not exclude the development of the disease (Boecker, 2006).

The most relevant risk factors for breast cancer development include (Table 1):

Age: The incidence of breast cancer increases with age. In some countries there is a flattening of the age-incidence curve after menopause (Dixon, 2006).

Geographical variation: Age adjusted incidence and breast cancer mortality has an heterogeneous geographic distribution, differing markedly from country to country (Dixon, 2006), as depicted in Figure 1.

Age of menarche and menopause: The risk of developing breast cancer increases in women with early menarche and late menopause (*i.e.*, a large fertile period). Women that have a natural menopause after the age of 55 are twice as likely to develop breast cancer when compared to women who have a natural menopause under the age of 45 (Dixon, 2006).

Age of first pregnancy: Both nulliparity and late age at first birth increase breast cancer risk. The highest risk group are women who have their first child after the age of 35, even higher than nulliparous women (Dixon, 2006).

Family history: Although only 5% to 10% of breast cancer cases have an hereditary predisposition, the lifetime risk of developing breast cancer in these women is 40% to 80% (Pruthi et al., 2007). Specific chromosomal alterations have been related to breast cancer risk. The *BRCA1* gene is mapped in the long arm of chromosome 17 and the *BRCA2* gene is located on the chromosome 13, and several types of mutations in different segments of these genes have been identified (Rosen, 2009). *BRCA* mutation carriers have 50% to 85% increased risk

of developing breast cancer by the age of 70 when compared with general population (about 11% risk by age 70) (Carroll et al., 2008). *BRCA1* may account for up to 45% of cases of hereditary breast carcinoma as well as for nearly 90% of patients with combined breast and ovarian cancer. Two other genes have been associated with familiar syndromes involving breast cancer: *TP53* (Li-Fraumeni syndrome, associated to brain tumors and sarcomas) and *PTEN* (Cowden syndrome, associated with other benign tumors of the breast and thyroid cancer), but they are both rare (Dixon, 2006).

Previous benign breast disease: There are several morphologic conditions that increase the risk of developing breast cancer. Women with palpable cysts, complex fibroadenomas, duct papillomas, sclerosing adenosis and florid epithelial hyperplasia have a higher risk of breast cancer (1.5 – 3 times) when compared with women without these alterations. Women with severe atypical epithelial hyperplasia have up to five times increased risk of developing breast cancer than women who have no proliferative lesions (Dixon, 2006).

Radiation-related: In teenage girls, the exposure to radiation during II World War doubled the risk of developing breast cancer up to the subsequent 30 years. A contemporary risk group consists of women who were treated for Hodgkin lymphoma with mantle type radiotherapy in their teens or early 20s. These women require screening earlier than the general population, since they have a significantly higher risk of developing the disease (Dixon, 2006; Pruthi et al., 2007).

Lifestyle:

- ✓ **Hormone Replacement Therapy:** Evidence suggests that the use of hormone replacement therapy (HRT) reduces the risk of coronary heart disease and osteoporosis by about 50%. However, it increases the risk of breast cancer by 30% to 40%, when used for five years or longer. In current users of HRT and those who have used HRT in the previous one to four years, relative risk (RR) increases by 1.023 (1.011 – 1.036) for each year of use. When a combination of estrogen and progesterone preparations is used, the risk of breast cancer is apparently higher. Interestingly, it has been suggested that increased surveillance among women taking hormones accounted for the increased risk in several studies. This is supported by the fact that a higher RR is associated with *in situ* rather than invasive cancer (Boecker, 2006).

- ✓ **Weight:** In postmenopausal women, obesity has been shown to increase breast cancer risk by 50%, while before menopause it is associated with a slightly decreased risk (Dixon, 2006; Pruthi et al., 2007). Conversely, weight loss or maintenance of ideal body weight and moderate physical activity has been shown to reduce the risk of breast cancer in adult women by approximately 30% (Pruthi et al., 2007).
- ✓ **Oral contraceptives:** Early studies on the relationship between the use of oral contraceptives (OC) and the risk of breast cancer provided controversial results. Nevertheless, later studies suggest an association between long-term use of OC and women on HRT and breast cancer risk (Dixon, 2006).
- ✓ **Diet:** There is a correlation between the incidence of breast cancer and fat intake at the population level. However, the true relationship between the two does not seem to be particularly strong or consistent. The same holds true as far as alcohol consumption is concerned, although the link between alcohol consumption and the risk of breast cancer could be associated with dietary factors other than alcohol (Dixon, 2006).

Table 1: Established and probable risk factors for breast cancer (adapted from Dixon, 2006)

Factor	Relative Risk (RR)	High Risk Group
Age	>10	Elderly
Geographical location	5	Developed countries
Age at menarche	3	Before age 11
Age at menopause	2	After age 54
Age at 1 st full pregnancy	3	First child in early 40s
Family history	>2	Breast cancer in 1 st degree relative when young
Previous benign disease	4-5	Atypical hyperplasia
Cancer in other breast	>4	
Premenopausal	0.7	Body mass index >35
Postmenopausal	2	Body mass index >35
Alcohol consumption	1.3	Excessive intake
Exposure to radiation	3	Abnormal exposure > age 10
Oral contraceptives	1.24	Current use
Combined HRT	2.3	Use for > 10 years

3. ETIOLOGY

Breast cancer is a highly heterogeneous disease at a clinical and pathological levels, presenting in several histological and molecular forms (Dixon, 2006; Polyak, 2007; Bertucci and Birnbaum, 2008; Geyer et al., 2009). To understand the different forms of breast cancer it is important to determine the cell of origin, the molecular alterations, the identification of susceptibility genes and the classification of tumors (Polyak, 2007; Bertucci and Birnbaum, 2008). The initiation of breast cancer is due to transforming events (genetic or epigenetic) occurring in a single cell. Subsequent accumulation of additional genomic changes, combined with clonal expansion and selection, lead to tumor growth and progression (Polyak, 2007; Bertucci and Birnbaum, 2008).

The mammary gland is a unique organ that undergoes extensive remodeling and differentiation, even in adults. In each menstrual cycle, hormonal changes induce cyclic modifications of proliferation in the mammary epithelium. Based on these observations, the existence of normal human adult mammary stem cells has been proposed, and their existence has been first suggested by transplantation studies conducted by DeOme et al., who were able to find the need for cell proliferation and cell replacement at various time points in the mammary gland (Polyak, 2007; Cariati and Purushotham, 2008). The existence of mammary stem cells is also indicated by the expansion and regenerative ability of the gland during puberty and successive reproductive cycles, the existence of two different cell lineages arising from a common progenitor, and the replacement of cells that are shed from the epithelium into the lumen during routine cell turnover (Cariati and Purushotham, 2008). However, the cellular identity and molecular characteristics of these cells have not been clearly defined, yet (Polyak, 2007).

Mammary stem cells renew themselves and differentiate, generating rapidly dividing progenitors. These, in turn, generate differentiated cells of the mammary gland epithelial lineages: the luminal and myoepithelial lineages (Bertucci and Birnbaum, 2008; Hwang-Verslues et al., 2008). The adult mammary gland is composed of at least three cell lineages. These include **myoepithelial cells** that form the basal layer of the ducts and alveoli, **ductal epithelial cells** that line the lumen of the ducts, and **alveolar epithelial cells** responsible for the synthesis of milk proteins. (Polyak, 2007; Hwang-Verslues et al., 2008).

The model of clonal evolution of tumors explains some of the key characteristics of cancer growth but it is probably too simplistic. Building upon the clonal evolution theory, there are two different models for how tumors develop and progress through unlimited cell division: the stochastic and hierarchical models of tumor development. The stochastic model postulates that all cells in a tumor have equal potential to be tumorigenic (*i.e.*, any cell from that tumor has an equal probability to form a new tumor with characteristics similar to the primary one). The hierarchical model postulates that only a subset of cells in a tumor has this tumorigenic capacity, whereas the rest of the tumor is composed by cells with varying degrees of differentiation which cannot regenerate the tumor on their own (Morrison et al., 2008).

This latter model is in concordance with the cancer stem cell hypothesis, in which the cancer stem cells (but not the differentiated cells that make up the bulk of the tumor) are responsible for tumor self-renewal (Morrison et al., 2008). While the exact etiology of breast cancer is unknown, cancer is thought to originate in these stem cells or in progenitor cells that have acquired self-renewal properties.

Whether a tumor comes from a stem cell or from a progenitor cell may be one of the main reasons for breast cancer heterogeneity. Tumors have been characterized as heterogeneous, composed of several types of differentiated and undifferentiated cells. There is emerging evidence that some solid tumors may contain a cancer cell hierarchy similar to that observed in the normal tissue from which they arose, with a cancer stem cell producing a progeny with limited replication potential (Cariati and Purushotham, 2008; Morrison et al., 2008). Cancer stem cells may therefore drive the growth and spread of the tumor.

It has been suggested that cancer stem cells may arise in either one of two ways. In the first case, oncogenic mutations in normal stem cells may produce alterations in the mechanisms that cause constraints on normal stem cell expansion, such as stem cell dependence on the niche (either by expansion of the niche itself or by acquisition of independence from niche signaling). In the second situation, oncogenic mutations allow transit-amplifying cells to continue proliferating without entering a post-mitotic differentiated state, therefore allowing aberrant activation of stem cell self-renewal mechanism in these cells (Cariati and Purushotham, 2008).

4. DIAGNOSIS AND GRADING

The multidisciplinary approach of newly diagnosed breast cancer gathers together a team of breast experts, including radiology, pathology, surgery and medical oncology specialists.

Breast Imaging: Mammography is the most established method for imaging the breast and it is the primary tool for breast disease evaluation. If a woman is asymptomatic, she may undergo screening mammography. Symptomatic women (*e.g.*, with palpable abnormality, skin changes, or nipple discharge) undergo diagnostic mammography.

Ultrasound is frequently used as an accessory tool to help diagnostic mammography. Most women with a palpable abnormality will undergo a focused echographic examination involving the area of clinical concern. Also, ultrasonography is often used to further characterize a mammographic abnormality and it is a common guide for breast intervention. The goal of screening mammography is to detect breast cancer in an early stage, before it becomes symptomatic and metastasizes. The overall sensitivity of mammography for breast cancer detection is approximately 85%. However, studies that evaluate women with *BRCA* mutations and dense breasts report sensitivities of only 38% to 55% (Pruthi et al., 2007).

Breast biopsy techniques:

- ✓ **Core needle biopsy:** A large-bore automated cutting needle is used to remove several (3 to 5) solid cylindrical tissue samples (“cores”). For adequate sampling, a 14-gauge or larger needle is required. These procedures are performed while guided by ultrasonography or stereotactic imaging. In most cases this is the preferred method for biopsy, since it usually allows for tumor grading and hormonal receptor analysis (or eventually HER2 status), both of which are important in formulating the patient’s treatment plan (Nemec et al., 2007).
- ✓ **Fine needle aspiration (FNA):** A smaller-bore (usually 18- or 20-gauge) needle is used to obtain cytologic samples from a suspicious breast mass. This is technically easier to perform and, in many cases, this minimally invasive procedure allows for the collection of representative material for cytological

evaluation, permitting the diagnosis of malignancy, although it may not provide sufficient cells for more detailed studies. Thus, in general a second (core) biopsy specimen is required for additional studies before a definitive treatment can be planned. Moreover, the accuracy of FNA in the diagnosis of breast malignancy depends on the cytopathologist proficiency both in performing the aspirate and the cytomorphological analysis (Jeronimo et al., 2003; Nemeč et al., 2007). As a consequence, false negative rates for this procedure range from 5 to 30%, and this could represent a major limitation for the identification of small preinvasive lesions and well-differentiated tumors. This problem could be overcome by coupling the evaluation of cellular morphology with analyses of tumor associated DNA alterations (Jeronimo et al., 2003).

- ✓ **Excisional biopsy:** This procedure is performed by a surgeon in the operating room, to remove the entire mass or suspicious area. Excisional biopsy requires preoperative wire localization if the lesion is not palpable (Nemeč et al., 2007).

Molecular studies have confirmed that grading the histological differentiation of the tumor provides very important information. It has been demonstrated that grade, more than any other clinicopathological parameter or tumor intrinsic characteristic, is associated with type, pattern and complexity of molecular changes seen in breast cancer and its precursors (Dixon, 2006; Geyer et al., 2009).

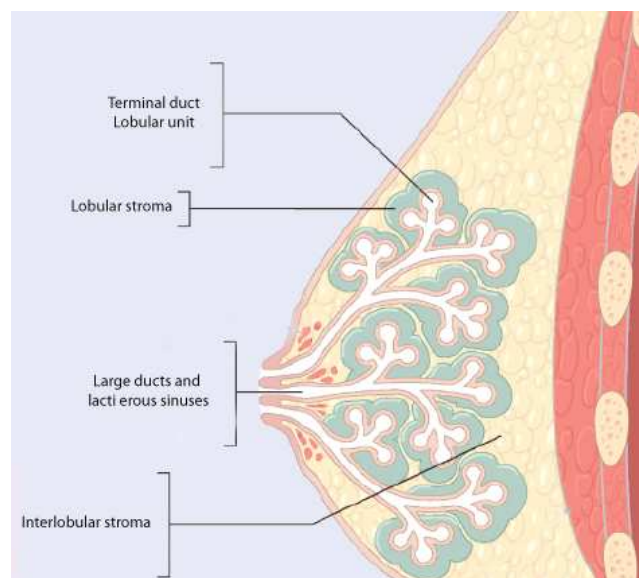


Figure 4: Normal breast anatomy (Adapted from Kumar, 2005)

Breast cancers are derived from the epithelial cells that are found in the terminal duct lobular unit (Figure 4). When the tumor cells are restricted to the basement membrane of the elements of the terminal duct lobular units and the draining duct they are classified as *in situ* or non-invasive. When dissemination of cancer cells occurs outside the basement membrane of the ducts and lobules into the surrounding adjacent normal tissue occurs they are called invasive breast cancers. Both *in situ* and invasive cancers have characteristic patterns that can be classified (Dixon, 2006). The most common classification of invasive breast cancers divides them into ductal or lobular types, as the belief is that ductal carcinomas arise from the ducts, and lobular carcinomas from lobules. In fact, both lobular and ductal carcinomas originate in the terminal duct lobular unit, so this terminology is somewhat inappropriate, although it is still in use (Dixon, 2006).

The *Scarff Bloom Richardson* grading system (Table 2) assesses the degree of tumor differentiation. It combines the nuclear grade (changes in nuclear size, uniformity, and nucleolar characteristics), tubule formation (percentage of cancer composed of tubular structures) and mitotic rate (rate of cell division) of the tumor.

Table 2: Modified Bloom-Richardson Histologic Grading (adapted from Rosen, 2009)

Tubule Formation	Score
> 75% of tumor has tubules	1
10-75% of tumor has tubules	2
less than 10% tubule formation	3
Nuclear Pleomorphism	Score
Small, uniform cells, similar to normal duct cell nuclei	1
Moderate increase in size and variation	2
Very large nuclei, usually vesicular and prominent nucleoli	3
Mitotic Count (per 10 hpf¹ with 40x objective and field area of 0.196mm²)	Score
Up to 7 mitoses	1
8 - 14 mitoses	2
15 or more mitoses	3

¹ Hpf: high power magnification field

To each of these features is assigned a score ranging from 1 to 3, and the sum determines the final grade. Scores 3 to 5 represent well differentiated (grade I), scores 6 and 7, intermediately (grade II), and scores 8 to 9, poorly differentiated (grade III) tumors (Kumar, 2005; Rosen, 2009).

The TNM (Tumor-Node-Metastasis) staging system classification includes four classifications: clinical, pathologic, recurrence, and autopsy. The clinical classification (**cTNM**) is used to make local/regional treatment recommendations. It is based solely on evidence gathered before initial treatment of the primary tumor: physical examination, imaging studies (including mammography and ultrasound), and pathologic examination of the breast or other tissues obtained from biopsy as appropriate to establish the diagnosis of breast cancer (Singletary and Connolly, 2006).

Pathologic classification (**pTNM**) is used to assess prognosis and to make recommendations for adjuvant treatment. It describes the anatomic extent of cancer and is based on the premise that the choice of treatment and the chance of survival are related to the extent of the tumor at the primary site (T1 to T4), the absence or presence of tumor in regional lymph nodes (N0 to N3), and the absence or presence of metastasis beyond the regional lymph nodes (M0 or M1) (Singletary and Connolly, 2006; Greene and Sobin, 2008). The currently used TNM staging system for breast cancer, based on hematoxylin and eosin (H&E) staining, description of histologic type, grading and evaluation of resection margins, maintains the dual approach of pretreatment clinical staging complemented by postsurgical histopathologic examination (Pruthi et al., 2007; Pestalozzi and Castiglione, 2008).

However, the fact that the TNM system is well established as a prognostic tool does not reflect the biological heterogeneity of breast cancer. For instance, it fails to explain why about one third of the women with node negative breast cancer will eventually develop distant metastases (Pruthi et al., 2007).

5. STAGING AND PROGNOSTIC INDICATORS

Staging systems take into account the extent of tumor spread in the body. They serve as critical guides to physicians in deciding appropriate treatment strategies and in discussing prognosis (Pruthi et al., 2007).

Traditionally, determination of tumor size, histological type and grade, lymph node status, vascular invasion, endocrine receptor status (estrogen and progesterone receptors), and human epidermal growth factor receptor 2 (HER2) status have driven prognostic predictions and adjuvant therapy recommendations for patients with early stage breast cancer (Cianfrocca and Gradishar, 2009; Geyer et al., 2009).

The 30-year survival rate of women with specific histologic types of invasive carcinomas (tubular, mucinous, medullary, lobular, and papillary) is greater than 60%, when compared with women with cancers of no special type (less than 20%) (Kumar, 2005).

When tumor cells are seen within vascular spaces (either lymphatics or small capillaries) surrounding tumors, these findings are associated with the presence of lymph node metastases. It is also a poor prognostic factor in women without lymph node metastases. The presence of tumor cells in lymphatics of the dermis is strongly associated with the clinical appearance of inflammatory cancer and indicates a very poor prognosis (Kumar, 2005).

Routine staging clinical examinations include physical exams, full blood counts and routine chemistry (liver enzymes, alkaline phosphatase, calcium and assessment of menopausal status). In patients with higher risk (with four or more positive axillary nodes, T4 tumors or with clinical suspicious of metastasis), chest X-ray, abdominal ultrasound and isotopic bone scan are appropriate (Pestalozzi and Castiglione, 2008).

Studies have shown that vascular invasion is an important prognostic factor, particularly in node-negative breast cancer (Pruthi et al., 2007; Pestalozzi and Castiglione, 2008).

The determination of hormonal status by immunohistochemistry of both estrogen receptor (**ER**) and progesterone receptor (**PgR**) is routine practice. The

report of immunohistochemical results for ER and PgR should include the percentage of ER- and PgR-positive cells. The hormonal status is no longer included as a prognostic factor, according to the St Gallen Consensus, but is the most relevant predictive factor for the choice of treatment. The ER expression is correlated to the treatment effect because of the correlation of better response to **tamoxifen** in tumors with high estrogen receptor levels (Pruthi et al., 2007; Pestalozzi and Castiglione, 2008).

Amplification of the **HER2** gene (mapped at 17q21) and/or overexpression of its protein product have been found in up to 25% to 30% of human breast cancers (Kumar, 2005; Murphy and Modi, 2009). Immunohistochemical determination of HER2 receptor expression should be performed at the same time for treatment planning, since its amplification or overexpression has been associated with poor survival in patients with axillary lymph node metastases. When results of immunohistochemistry are ambiguous, *in situ* hybridization (either fluorescent - FISH - or chromogenic - CISH) to determine **HER2** gene amplification should be performed. HER2 status is routine because it is a predictive marker for response to chemotherapy and agents directed against HER2, such as the monoclonal antibody **trastuzumab** and the tyrosine kinase inhibitor **lapatinib** (Pruthi et al., 2007; Pestalozzi and Castiglione, 2008; Murphy and Modi, 2009).

Proliferation can be measured by flow cytometry or by immunohistochemical detection of cellular proteins (*e.g.*, cyclins, Ki-67) produced during the cell cycle. Mitotic counts are also included as part of the standard grading system. Tumors with high proliferation rates have a worse prognosis, but the most reliable method to assess proliferation has not yet been established. (Kumar, 2005).

Over the last years, breast cancer has been the most studied epithelial neoplasia through molecular biology techniques. Therefore, predictive markers and novel therapeutic targets are expected to emerge in the near future (Geyer et al., 2009).

II. THE MOLECULAR BIOLOGY OF BREAST CANCER

1. GENETICS

Cancer is thought to be, in its essence, a genetic disease. Genetic alterations involved in breast cancer tumorigenesis include the activation of growth-promoting protooncogenes (*e.g.*, *Cyclin D1*), the inactivation of growth-inhibiting tumor suppressor genes (*e.g.*, *RB* and *TP53*), alterations in cell-cycle control genes (*e.g.*, *BRCA1* e *BRCA2*), alterations in genes involved in DNA repair (*e.g.*, *APC*), and in cell adhesion and invasion (*e.g.* *CDH1*) (Kumar, 2005; Lo and Sukumar, 2008). Loss of *RB* function could lead to aggressive proliferation and resistance to anti-estrogen hormonal therapy; *BRCA1* e *BRCA2* germline mutations are associated with hereditary breast cancer and *TP53* is associated with Li-Fraumeni syndrome (Hwang-Verslues et al., 2008).

Mutations in protooncogenes are considered dominant, because only one mutant allele suffices to transform cells, even if a normal allele is still present. However, in tumor suppressor genes, both alleles need to be inactivated in order for the transformation to occur. Genes that regulate apoptosis may be dominant, as are protooncogenes, or they may behave as tumor suppressor genes. A disability in DNA repair genes can also predispose to neoplasia, due to the effect that these genes have in cell proliferation or survival by influencing the ability of the organism to repair damage in other genes, including protooncogenes, tumor suppressor genes, and genes that regulate apoptosis. Usually, those genes need bi-allelic inactivation in order to induce genomic instability (Kumar, 2005).

Carcinogenesis is a multistep process at both the phenotypic and genomic levels. A malignant neoplasm has several phenotypic attributes, such as excessive growth, local invasiveness, and the ability to form distant metastases. These characteristics are acquired in a stepwise fashion, a phenomenon called tumor progression. At the molecular level, progression results from accumulation of genomic lesions that in some instances are favored by defects in DNA repair (Kumar, 2005).

2. EPIGENETICS

Cell transformation is, in many cases, due to genetic alterations (mutations, translocations, loss of heterozygosity, etc). However, not all cellular changes can be explained through this pathway. Several tumor suppressor genes can be silenced via another mechanism – epigenetic alterations. The importance of epigenetics was only recognized over the last two decades, following Feinberg and Vogelstein's observation that abnormal DNA methylation events could be associated with cancer (Jones and Baylin, 2007; Esteller, 2008).

Epigenetics can be defined as heritable changes in gene expression that do not derive from alterations in the DNA sequence (Feinberg and Tycko, 2004; Jones and Baylin, 2007; Esteller, 2008; Liu et al., 2008), and several main mechanisms have been identified: chromatin and nucleosome remodeling (via posttranslational modifications of histone proteins), (Hebbes et al., 1988; Jones and Baylin, 2007; Mulero-Navarro and Esteller, 2008), DNA methylation (Esteller, 2007; Jones and Baylin, 2007; Esteller, 2008; Mulero-Navarro and Esteller, 2008), including genomic imprinting (Feinberg and Tycko, 2004), and microRNA (Esteller, 2008; Heneghan et al., 2009). The interplay between those epigenetic events modulates chromatin conformation and gene expression. Alterations in gene expression induced by epigenetic deregulation lead to a cellular growth advantage, which results in the progressive uncontrolled growth of a tumor (Jones and Baylin, 2007; Lo and Sukumar, 2008).

Little is known yet about the histone modification patterns in human cancer (Feinberg and Tycko, 2004; Esteller, 2007). Initial studies of chromatin remodeling focused on histone acetylation, a reversible biochemical process that confers either open or condensed chromatin conformations in order to alter gene expression (Jones and Baylin, 2007; Liu et al., 2008). Histone modifications mediated by various histone tail-modifying enzymes collaborate with or without DNA methylation to communicate with chromatin remodeling factors, which adapt chromatin structure to the open euchromatin (active transcription) or the closed heterochromatin (repressed transcription) states (Lo and Sukumar, 2008). Both acetylation and methylation of histones affect several nuclear processes, such as DNA repair, DNA replication, and gene transcription (Esteller, 2008). DNA methylation, histone covalent modifications, and nucleosome remodeling all

intervene in development and differentiation (Mulero-Navarro and Esteller, 2008), hence its importance in carcinogenesis.

More recently, a family of short, 22 nucleotide non-coding RNAs – microRNAs – has been described as playing a key regulatory role in gene expression. Through the binding to 3'UTR of target mRNAs, these molecules can repress protein expression either by inhibiting translation or promoting mRNA degradation. Patterns of microRNA expression are tightly regulated and play important roles in cell proliferation, apoptosis, and differentiation. These processes are commonly deregulated in cancer, thus implicating miRNAs in carcinogenesis. The first evidence of involvement of miRNAs in malignancy came from the identification of a translocation-induced deletion in B-cell chronic lymphocytic leukemia. Loss of miR-15a and miR-16-1 from this *locus* results in increased expression of the antiapoptotic gene *bcl2*. The number of human genes known to lose activity as a result of the binding of a miRNA to the untranslated regions of the mRNA is growing rapidly. Recent studies have shown that profiles of miRNA expression differ between normal and tumor tissues and among tumor types, correlating with various cancers. Interestingly, miRNAs are thought to function either as tumor suppressors or proto-oncogenes in a number of human malignancies, including breast cancer (Esteller, 2008; Heneghan et al., 2009).

Imprinting refers to conditioning of the maternal and paternal genomes during gametogenesis, in which a specific parental allele is more abundantly, or exclusively, expressed in the offspring (Feinberg and Tycko, 2004), meaning that the other parental allele is relatively or absolutely silenced. This is maintained in part, by methylated regions within or near imprinted genes (Feinberg and Tycko, 2004). One of the earliest findings of loss of imprinting (LOI) was made by Feinberg and colleagues in the early 1990's when they demonstrated pathological bi-allelic expression of **IGF2** in Wilms tumors.

✓ **DNA methylation**

Currently, the best-known epigenetic modification in human cells is DNA methylation (Esteller, 2007; Jones and Baylin, 2007; Esteller, 2008; Mulero-Navarro and Esteller, 2008). Methylation occurs in cytosines that precede guanines - CpG dinucleotides - which are not randomly distributed along the genome. Instead, the CpG-rich regions (also called CpG islands) span the 5' end

regulatory region of many genes (Kumar, 2005; Jones and Baylin, 2007; Esteller, 2008; Liu et al., 2008; Mulero-Navarro and Esteller, 2008). DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs), which add of a methyl group to the 5-carbon position of cytosine in CpG dinucleotides (Figure 5), and is generally associated with gene silencing (Liu et al., 2008; Lo and Sukumar, 2008).

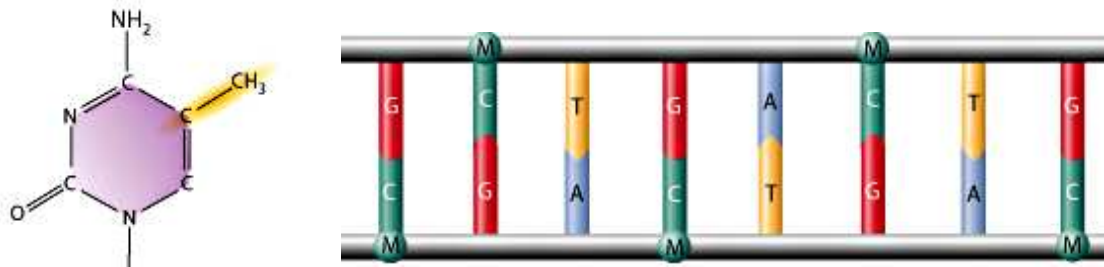


Figure 5: DNA methylation. On the left, the addition of a methyl group (yellow) to de cytosine molecule (purple). On the right a schematic representation of a DNA strand, with methylated cytosines (adapted from <http://www.artksthoughts.blogspot.com>).

In DNA from normal cells, methylation occurs in about 3-6% of all cytosines. Indeed, CpG islands are usually unmethylated in normal cells and *de novo* methylation seldom occurs in normal tissues (Jones and Baylin, 2007; Esteller, 2008; Liu et al., 2008; Mulero-Navarro and Esteller, 2008). The unmethylated status corresponds to the ability of CpG-island containing genes to be transcribed in the presence of the necessary transcriptional activators (Esteller, 2007). By contrast, repetitive genomic sequences are heavily methylated. The maintenance of this DNA methylation pattern might have a role in the protection of chromosomal integrity, by preventing chromosomal instability, translocations and gene disruption (Esteller, 2007; Lopez et al., 2009).

In cancer cells, the methylation pattern is altered. Promoter hypermethylation and global genomic hypomethylation coexist (Ting et al., 2006; Mulero-Navarro and Esteller, 2008). Global hypomethylation has been found to increase with age and is linked to genomic instability and activation of protooncogene expression (Lo and Sukumar, 2008). DNA methylation is linked to tissue-specific gene silencing, and differences between cancer and normal tissues were first identified by Vogelstein and Feinberg in the early 80's. They found that a substantial proportion of CpG islands that were methylated in normal tissues, were unmethylated in cancer cells. Hypomethylation of DNA can lead to gene

activation and the CpG islands that were hypomethylated in cancer cells activate protooncogenes, such as *HRAS* (Feinberg and Tycko, 2004).

Concordant with the hypomethylation events, gene-specific DNA hypermethylation has been shown to occur in most human cancers, including breast cancer. Certain tumor suppressor genes may be inactivated through hypermethylation of the respective promoter sequences (Kumar, 2005; Lo and Sukumar, 2008; Lopez et al., 2009).

The initial reports on hypermethylation of CpG islands concerned the *RB* gene in some retinoblastomas (Esteller, 2008; Mulero-Navarro and Esteller, 2008), followed by findings of DNA hypermethylation in several other tumor suppressor genes. Other genes that have CpG islands in their promoter region and have been shown to be subject to aberrant hypermethylation include those whose protein products are involved in cell cycle regulation, DNA repair, apoptosis, cell adhesion, and angiogenesis (Esteller, 2008; Lopez et al., 2009). Among these are DNA repair genes such as *hMLH1* (in colon cancer), *MGMT* (in gliomas) and *VHL* (in renal cell cancer) (Kumar, 2005; Mulero-Navarro and Esteller, 2008).

The E-cadherin (*CDH1*) gene is located at the chromosome 16q22.1. It encodes a cell-surface adhesion protein which plays a role in maintaining cell-cell adhesion in epithelial tissues. Evidence shows that loss of expression of E-cadherin contributes to increased proliferation, invasion and metastasis in breast cancer. Mutations and deletions play an important role in loss of CDH1 expression and function (Yang et al., 2001). However, several studies demonstrate that epigenetic silencing of the *CDH1* gene occurs in breast cancer through several mechanisms, including aberrant promoter methylation (Droufakou et al., 2001), and it has been associated with a specific type - lobular carcinomas (Parrella et al., 2004).

According to Knudson's 'two-hit' model, inactivation of a tumor suppressor gene requires loss-of-function in both copies of the gene. Some of the methylated genes identified in human cancers are classic tumor suppressor genes, in which a mutation of one of the alleles might be inherited. The second could be the epigenetic silencing of the remaining wild-type allele of the tumor suppressor gene (Esteller, 2008; Lo and Sukumar, 2008), as represented in Figure 6.

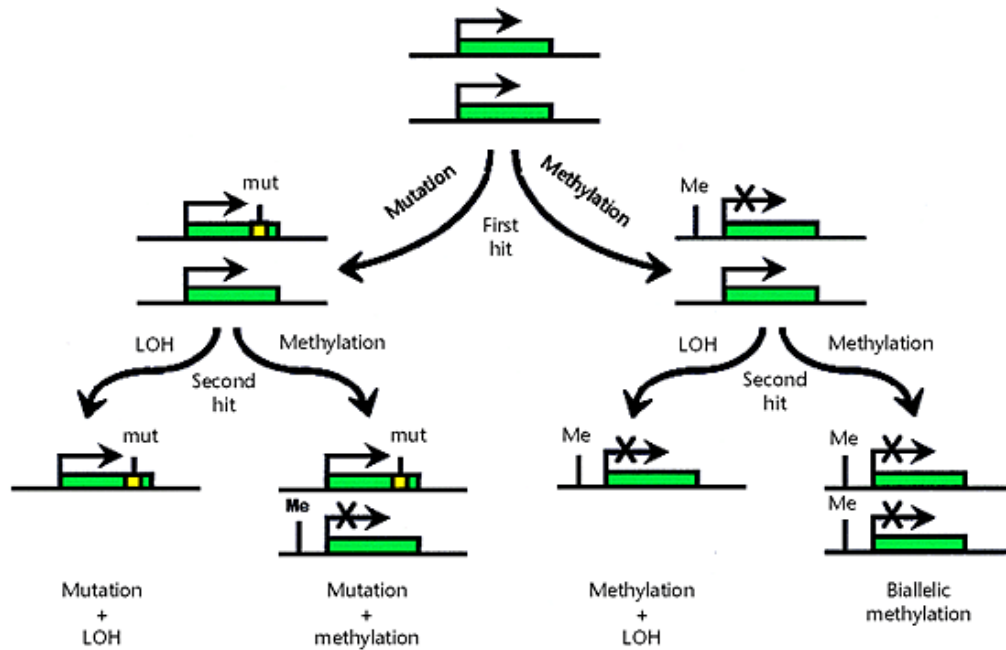


Figure 6: Contribution of methylation for the inactivation of TSG (adapted from Allis, 2007)

Epigenetic alterations are one of main driving mechanisms leading to breast cancer. Indeed, some well-known tumor suppressor genes, such as *p16/INK4a*, *APC* and *BRCA1* which are mutationally inactivated in the germline, occasionally lose function of the remaining functional allele in breast epithelial cells through DNA hypermethylation. Hence, novel TSGs can be identified using DNA methylation as a marker. Hypermethylated genes identified from breast neoplasms now form a long list. Their biological functions encompass cell cycle regulation (*p16/INK4a*, *Cyclin D2*), apoptosis (*APC*, *DAPK1*, *TWIST*), DNA repair (*GSTP1*, *MGMT*, *BRCA1*), hormone regulation (*ER α* , *PR*), cell adhesion and invasion (*CDH1*, *APC*), angiogenesis (*THBS1*), cellular growth-inhibitory signaling (*RAR β* , *RASSF1A*, *HIN1*), among others (Hoque et al., 2006; Hinshelwood and Clark, 2008; Jeronimo et al., 2008; Lo and Sukumar, 2008).

Over the last few years, the mapping of genes in which promoter CpG islands are hypermethylated in cancer has been increasing. This search revealed unique profiles of hypermethylation that define each neoplasia. Methylation can be, therefore, used as a biomarker of cancer cells. For instance the *GSTP1* gene is hypermethylated in 80 to 90% of patients with prostate cancer, but seldom in benign hyperplastic prostate tissue (Esteller, 2007; Esteller, 2008).

In breast, this methylation patterns have been developed as biomarkers for early detection and subtype classification of breast tumors, as predictors for risk assessment and monitoring prognosis, and as indicators of susceptibility or response to therapy (Esteller, 2008; Lo and Sukumar, 2008). Presence of methylated DNA in several types of biological fluids such as nipple duct fluids and needle aspirates of the breast might also be used to predict breast cancer development (Agrawal et al., 2007).

III. GENES

1. CYCLIN D2

Genetic analysis of human tumors revealed that some of the genes most often altered in cancer are those involved in the control of the G1 (preparation for DNA synthesis) / S (DNA synthesis) transition of the cell cycle, a time when cells become committed to a new round of cell division. G1 is the initial phase of the cell cycle when cells must acquire all the necessary information to proceed safely into the next phase, S, when their genetic dowry has to be faithfully duplicated (Ortega et al., 2002; Chiles, 2004; Azzato et al., 2008).

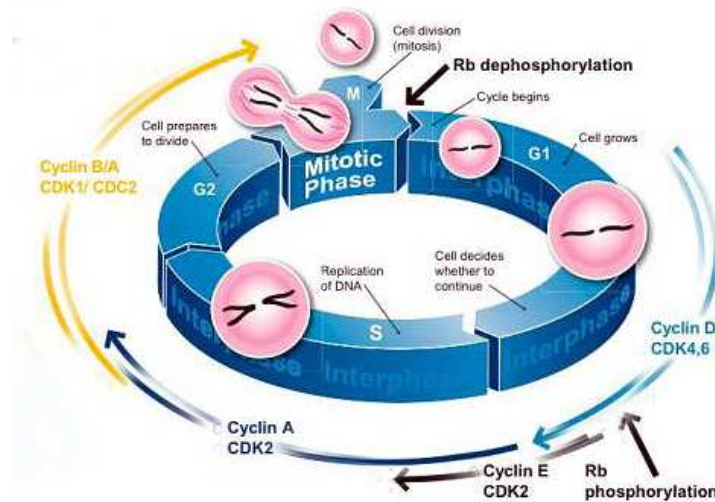


Figure 7: Cyclins and cell cycle regulation (adapted from <http://www.sapphirebioscience.com>)

The D-type cyclins (including *Cyclin D2*) are involved in regulation of the G1 to S transition. Their critical function is to activate cyclin-dependent kinases (CDKs) *CDK4* and *CDK6*, leading to the phosphorylation of *RB*, the retinoblastoma protein. This, in turn, leads to release of transcription factors such as *E2F* from RB-mediated repression, which then activate transcription of genes involved in DNA synthesis and thus trigger the onset of S-phase (Evron et al., 2001). This cascade has been found to be altered in more than 80% of human neoplasias, either by mutations within the genes encoding these proteins or in their upstream regulators (Sherr, 1995; Ortega et al., 2002; Chiles, 2004).

Interactions of cyclins with CDKs play an important role in regulating the cell cycle. CDKs promote phosphorylation of their target proteins, initiating progression of the cell cycle (Schlotter et al., 2008).

As regulatory subunits of CDKs, D-type cyclins are rate limiting controllers of G1 phase progression in mammalian cells. Cyclin D2 is a member of the D-type cyclins, implicated in cell cycle regulation, differentiation, and malignant transformation (Evron et al., 2001).

In a previous study, *CCND2* was found to be methylated in significant levels in breast cancer (46%). Normal breast cells were also analyzed, and it was confirmed that *CCND2* methylation was specific of tumor cells (Evron et al., 2001). In addition, high levels of methylation were also found in both lobular and ductal carcinomas of the breast (Fackler et al., 2003).

2. RASSF1A

The *RASSF1A* gene, expressed in normal tissues, is functionally involved in cell cycle control, microtubule stabilization, cellular adhesion, motility and also apoptosis. Therefore, depletion of *RASSF1A* is associated with loss of cell cycle control, accelerated mitotic progression and an increased risk for chromosomal defects which leads to genetic instability, enhanced cellular motility and with increased tumor susceptibility (Donninger et al., 2007; Peters et al., 2007). The *RASSF1A* gene encodes a protein similar to the RAS effector proteins, it is located at 3p21.3, and its loss is one of the most frequent events in several types of human solid tumors. Current data suggest that inactivation or altered expression of *RASSF1A* is involved in the malignant progression of certain human cancers, suggesting the tumor suppressor function of this gene (Liu et al., 2002; van der Weyden and Adams, 2007).

Tumor suppressor genes are classically defined by the aforementioned Knudson's 'two-hit' hypothesis. Loss of a *RASSF1A* allele is a frequent event in primary human cancers (Donninger et al., 2007) (Table 3). *RASSF1A* alleles can be inactivated by a combination of genetic and epigenetic mechanisms. Therefore, *RASSF1A* fulfills the Knudson 'two-hit' model. Hypermethylation of both alleles of the *RASSF1A* promoter has been shown to cause loss of expression of the gene (Donninger et al., 2007; Peters et al., 2007).

Several studies have reported inactivation of *RASSF1A* gene by aberrant promoter hypermethylation in a high percentage of human cancers, including prostate (Liu et al., 2002), renal (Peters et al., 2007), breast (Euhus et al., 2007; Jeronimo et al., 2008) and colorectal neoplasms (Agrawal et al., 2007).

RASSF1A methylation has the potential to be an ideal cancer biomarker. It occurs at moderate to high frequency in a very wide range of tumor types, yet it is comparatively rarely found in normal tissues (van der Weyden and Adams, 2007).

In breast cancer patients, *RASSF1A* methylation has been shown to be frequently detected not only in tissue samples, but also in other clinical samples, including up to 75% of serum DNA samples (Shukla et al., 2006) and 62% of fine needle aspirate washings (Jeronimo et al., 2008).

Table 3: Primary tumors containing *RASSF1A* promoter methylation (adapted from Donninger, 2007)

Tumor Type	Frequency ²	References
Lung SCLC	88%	Grote et al., 2006
Breast	81-95%	Yeo et al, 2005; Shinozaki et al., 2005
Prostate	99%	Jeronimo et al., 2004
Renal	56-91%	Yoon et al., 2001; Dreijerink et al., 2001
Colorectal	20-52%	Miranda et al., 2006; Oliveira et al., 2005
Neuroblastoma	83%	Lazcoz et al., 2006
Gastric	44%	Oliveira et al., 2005

Thus, methylation of *RASSF1A* is being considered for use in clinical practice as a diagnostic marker, for early tumor detection, and a prognostic marker, to predict the risk of cancer development from benign growths, to predict the prognosis of patients with a diagnosed tumor, or even as a marker for resistance to some treatments (van der Weyden and Adams, 2007).

² Frequency of *RASSF1A* promoter hypermethylation in each tumor type

3. *APC*

The **adenomatous polyposis coli** (*APC*) gene is a tumor suppressor that is located at 5q21. Its protein is an important component of the **Wnt** signaling pathway, which controls cell proliferation and differentiation in cells from the intestine, skin, immune system, bone and brain (Virmani et al., 2001; Aoki and Taketo, 2007).

APC inactivation has also been proposed to promote tumorigenesis through loss of cell adhesion (by the interaction with β -catenin, which links E-cadherin to α -catenin and the cytoskeletal actin), and cell migration and spindle formation (via microtubule stabilization). However, it is still unclear whether mutations in *APC* accelerate tumorigenesis through these mechanisms (Virmani et al., 2001; Aoki and Taketo, 2007).

Germline mutations of *APC* are present in colorectal carcinomas arising in familial adenomatous polyposis syndrome and somatic mutations initiate many of the sporadic colon cancers (Jin et al., 2001; Virmani et al., 2001).

Inactivation of *APC* may occur by way of multiple mechanisms, including allelic loss, gene mutation or methylation of CpG sites in promoter regions, and 60% occur within the mutation cluster region, a small region of exon 15 between codons 1286 and 1513. Whereas 18% of breast cancers have somatic mutations, mostly outside the mutation cluster region, mutations are rare or absent in other cancers, including non-small cell lung carcinomas (NSCLCs). However, allelic losses at 5q21 are frequent in breast and lung carcinomas, suggesting that mechanisms other than mutation may inactivate the other allele (Virmani et al., 2001).

Several studies refer CpG island hypermethylation as an event responsible for *APC* inactivation in human cancers, among which are colorectal (Agrawal et al., 2007), lung (Virmani et al., 2001; Esteller, 2005) and prostate (Esteller, 2005).

In breast cancer, high levels of *APC* hypermethylation have been described as well, and its detection was feasible not only in tumor samples, but also in plasma (Hoque et al., 2006), FNA washings (Jeronimo et al., 2008) and serum (Muller et al., 2003), the latter with prognostic significance. In the study by Hoque and co-workers, 43% of women whose primary cancer tissue harbored aberrant *APC* methylation, had this aberrantly methylated DNA detected in plasma.

OBJECTIVES

In previous studies from our research group, the feasibility of detection of DNA methylation at multiple promoters has been demonstrated in FNA washings from suspicious breast lesions (Jeronimo et al., 2003). Moreover, a defined gene panel was shown to augment the accuracy of breast cancer detection in the same type of samples (Jeronimo et al., 2008). Thus this study was designed to evaluate whether quantitative promoter methylation at 3 gene loci might carry prognostic information in addition to standard clinicopathologic parameters.

Specifically the aims of this study were:

- ✓ Determine and compare the methylation levels at the promoter region of three genes - *CCND2*, *RASSF1A* and *APC* - in samples obtained from FNA washings of malignant and benign breast lesions
- ✓ Evaluate the performance of the same gene panel as an ancillary tool to cytomorphologic diagnosis of malignant breast lesions
- ✓ Assess the prognostic value of the same epigenetic alterations in malignant breast lesions.

MATERIALS AND METHODS

I. PATIENTS

A total of 237 female patients with palpable suspicious breast lesions, consecutively submitted to FNA at the Portuguese Oncology Institute – Porto, Portugal, from 2002 to 2007, were enrolled in this study, following informed consent. Relevant clinical and pathological data was retrieved from the patient's clinical charts. These studies were approved by the IRB (Comissão de Ética) of Portuguese Oncology Institute – Porto.

II. CYTOLOGICAL PREPARATIONS

FNA biopsy was performed using a 23-gauge needle attached to a 10-ml syringe and inserted into a syringe holder. The aspirates were smeared on microscope slides and routinely stained for cytopathological evaluation.

Samples for methylation analysis were produced by washing the needle and syringe with 250 µl of PBS. The solution was spinned down, and the pellet was collected in a tube and stored at -80°C.

III. DNA EXTRACTION

DNA from FNA washings was extracted by the phenol-chloroform method, at pH 8, as described by Pearson et al (Pearson and Stirling, 2003)

Briefly, to digest the samples, 500 µL of buffer solution SE (75 mM NaCl; 25 mM EDTA), 20 µL de SDS 10% and 15 µL proteinase K (20 mg/mL) [Sigma, Germany] were added to each sample and incubated overnight at 55°C in a bath.

After digestion, extraction was completed with phenol/chloroform [Sigma, Germany]/ [Merck, Germany] in Phase Lock Gel™ tubes. After centrifugation (15 min at 14000 rpm), the upper aqueous phase was transferred to a new tube.

Precipitation followed through mixing 1000 µL of 100% cold ethanol, 165 µL of ammonium acetate and 2 µL of glycogen (5 mg/mL), and incubated overnight at -20°C. Finally, a washing was performed with 70% ethanol solution, dried and eluted in distilled water. Samples were stored at -20°C.

DNA concentration and quality were analysed by spectrophotometry in a NanoDrop system ND-1000 [NanoDrop Technologies, USA].

IV. METHYLATION ANALYSIS

1. BISULFITE MODIFICATION

This method allows for the assessment of the methylation status of individual CpG islands in genomic DNA. The key to determining methylated cytosines is based on the selective chemical reaction of sodium bisulfite with cytosine versus methylated cytosine residues. Treatment of DNA with sodium bisulfite results in sequence differences due to deamination of unmethylated cytosines to uracil whereby methylated cytosines (5-mC) remain unchanged (Derks et al., 2004; Esteller, 2005).

Sodium bisulfite conversion was performed as previously described (Clark et al., 1994). Briefly, 2 µg of genomic DNA, in a total of 20 µL, were denatured using 2 µL of 3M NaOH and 1 µL of salmon sperm DNA (10 mg/mL) [Invitrogen, CA, USA] for 20 min at 50°C.

The denatured DNA was diluted in 450 µL of a freshly prepared bisulfite reaction solution (sodium bisulfite 2.5M, hydroquinone 125 mM and NaOH 2M), and the mixture was incubated at 70°C for 3 hours in the dark. After incubation, the resulting bisulfite-modified DNA was desalted and purified using a vacuum manifold and a Wizard® DNA purification resin [Wizard DNA Clean-Up System; [Promega Corp., WI, USA] according to manufacturer's instructions. The eluted DNA was denatured in 5 µL of NaOH 3M (10 minute incubation at room temperature).

The modified DNA was precipitated by adding 350 µL of 100 % cold ethanol, 75 µL of ammonium acetate 7.5M and 1 µL of glycogen (5 mg/mL), and incubated overnight at -20°C. Finally, the precipitate was washed with 70% cold ethanol, left to dry, eluted in distilled water and stored at -80°C.

2. QMSP ANALYSIS

Methylation-specific PCR (MSP) is a method that allows for the distinction between unmethylated and methylated alleles in bisulfite-modified DNA, taking advantage of the sequence differences resulting from bisulfite modification, in which all uracil and thymine residues have been amplified as thymine and only 5-MeC residues have been amplified as cytosine (Esteller, 2007).

The modified DNA was used as a template for real-time fluorescence-based methylation-specific PCR (QMSP) using an Applied Biosystems 7000 Sequence Detector System (PerkinElmer Corp., Foster City, CA). Fluorogenic QMSP assays were carried out in 96-well plates.

In each well, a volume of 20 μ L of the reaction mix was added, which consisted in: 16.6mM ammonium sulfate; 67mM trizma preset; 6.7mM magnesium chloride; 10mM mercaptoethanol; 0.1% DMSO; 200 μ M each of dATP, dCTP, dGTP, and dTTP; 600nM of each primer; 0.4 μ L of Rox dye; 200nM of probe; 1 unit of Platinum Taq polymerase (Invitrogen, Carlsbad, CA), and 2 μ L of bisulfite-modified DNA as a template.

The primers and probes used for the each target gene (*APC*, *CCND2* and *RASSF1A*) and for the internal reference gene (β -actin - *ACTB*) are listed below and have been previously published (Eads et al., 2000; Lehmann et al., 2002). To determine the relative levels of methylated promoter DNA in each sample, the values of each target gene were normalized against the values of the internal reference gene to obtain a ratio that was then multiplied by 1,000 for easier tabulation [methylation level = (target gene/*ACTB*) \times 1000].

All amplifications were performed at 95°C for 2 minutes, followed by 50 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

PCR was done in separate wells for each primer/probe set, and each sample was run in triplicate. Each plate included multiple water blanks, which acted as a negative control, and a serial of dilutions of a positive control for constructing the corresponding calibration curve.

A given sample was considered positive when amplification was detected in at least two of the triplicates of the respective QMSP analysis. The QMSP threshold

was determined adjusting the best fit of the slope and R2 based on the respective calibration curve.

Table 4: MethyLight primer and probe sequences (Eads et al., 2000; Lehmann et al., 2002)

ACTB (GenBank: Y00474)	
Probe 6FAM 5´-3´TAMRA	ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA
Forward 5´-3´	TGG TGA TGG AGG AGG TTT AGT AAG T
Reverse 5´-3´	AAC CAA TAA AAC CTA CTC CTC CCT TAA
APC (GenBank: U02509)	
Probe 6FAM 5´-3´TAMRA	CCC <u>GTC</u> GAA AAC <u>CCG</u> <u>CCG</u> ATT A
Forward 5´-3´	GAA CCA AAA <u>CGC</u> TCC CCA T
Reverse 5´-3´	TTA TAT <u>GTC</u> <u>GGT</u> TAC <u>GTG</u> <u>CGT</u> TTA TAT
CCND2 (GenBank: AF518005)	
Probe 6FAM 5´-3´TAMRA	AAT <u>CCG</u> CCA ACA <u>CGA</u> <u>TCG</u> ACC CTA
Forward 5´-3´	TTT GAT TTA AGG ATG <u>CGT</u> TAG AGT <u>ACG</u>
Reverse 5´-3´	ACT TTC TCC CTA AAA ACC <u>GAC</u> TAC <u>G</u>
RASSF1A (GenBank: NM_007182.4)	
Probe 6FAM 5´-3´TAMRA	ACA AAC <u>GCG</u> AAC <u>CGA</u> <u>ACG</u> AAA CCA
Forward 5´-3´	<u>GCG</u> TTG AAG <u>TCG</u> GGG TTC
Reverse 5´-3´	CCC <u>GTA</u> CTT <u>CGC</u> TAA CTT TAA <u>ACG</u>

V. STATISTICAL ANALYSIS

The frequency of methylated and unmethylated cases, as well as the median and interquartile range of the methylation level for each gene in each group of tissue samples was determined. Methylation levels of the genes were expressed as continuous variables. Values were analyzed using non-parametric tests, i.e., the Kruskal-Wallis one-way analysis of variance, followed by the Bonferroni-adjusted Mann-Whitney U test when appropriate. For this comparison test among the three groups of tissue samples, the non-adjusted statistical level of significance of $p < 0.05$ corresponds to a Bonferroni adjusted statistical

significance of $p < 0.0167$. Receiver operator characteristic (ROC) curve analysis was used for each gene to determine the respective diagnostic performance, using the Area Under the Curve [AUC, with 95% confidence interval (CI)]. Histopathologic evaluation constituted the gold standard or reference test. Positivity for each methylated promoter was set as previously determined (Jeronimo et al., 2008) and quantitative estimates of validity were determined.

The prognostic significance of clinical and pathological variables (age, tumor grade, pathological stage and hormone receptor status) was assessed by constructing disease-specific and disease-free survival curves using the Kaplan-Meier method with log rank test (univariate test), and by a Cox-regression model comprising all variables (multivariate test). To test the prognostic significance of the methylation status for each gene, samples were categorized into two groups based on the methylation levels for that gene [using as a threshold the value of the percentile 75 (Henrique et al., 2007b)]. Disease-specific and disease-free survival curves were then constructed based on each of the three genes (univariate analysis). A Cox-regression model comprising both clinical and epigenetic variables was computed to assess the relative contribution of each variable to the assessment of follow-up status.

A P value smaller than 0.05 (two-sided) was considered to indicate statistical significance. Statistical analyses were carried out using a computer-assisted program (SPSS, version 11.0, Chicago, IL).

RESULTS

CHARACTERISTICS OF PATIENTS' POPULATIONS AND QMSP RESULTS IN BREAST CANCEROUS AND NON-CANCEROUS TISSUES

We tested FNA washing samples from 237 suspicious breast lesions, 148 of which were cytopathologically diagnosed as malignant and 37 as benign. In the remaining 52 cases no definitive cytomorphological diagnosis was rendered (this category includes “suspicious”, “inconclusive”, and “insufficient material” cases) (Table 5).

Table 5: Cytopathological classification of patient population of the 237 FNA washings

Cytologic diagnosis	Frequency (n)	Percent (%)	Cumulative %
Benign	37	15.6	15.6
Malignant	148	62.4	78.1
Not determined ³	52	21.9	100.0
TOTAL	237	100.0	100.0

Histopathological material for examination was available in 211 cases, comprising 178 malignant and 33 benign lesions (Table 6).

Table 6: Histopathological characteristics of patient population (n=237)

Histologic diagnosis	Frequency (n)	Percent (%)
Benign	33	13.9
Malignant		
<i>Carcinoma in situ</i>	3	1.3
Invasive ductal carcinoma	135	57.0
Invasive lobular carcinoma	9	3.8
Mixed type carcinomas	23	9.7
Other types	8	3.4
No histologic diagnosis	26	11

³ Not determined includes: insufficient, inconclusive and suspicious cases

The relevant clinical and pathological characteristics of the patients are given in Table 7.

Table 7: Clinical and pathological characteristics of patient population

	Malignant	Benign
Patients (n)	178	33
Age, years, median (range)	62 (29-92)	42 (18-77)
Tumor size, cm, median (range)	2.5 (0.45-9.5)	n.a. ⁴
Grade, n (%)		n.a.
1	31	
2	84	
3	57	
Not determined	6	
Stage, n (%)		n.a.
0	3	
I	41	
II	90	
III	35	
IV	4	
Not determined	5	
Hormonal Receptor status		n.a.
ER +	33	
ER -	142	
Not determined	3	
PgR +	62	
PgR -	113	
Not determined	3	

⁴ n.a.: Non applicable

QMSP was then performed in the 211 FNA washings corresponding to those cases with confirmatory histopathological diagnosis and the respective methylation frequencies and distribution of methylation levels are listed in Table 8. The frequency of promoter methylation was higher in malignant lesions for all genes, although a statistically significant difference was only observed for *APC* ($P = 0.003$). Breast cancers also displayed the highest methylation levels for all the analyzed genes. Statistically significant differences were depicted for *APC* and *RASSF1A*, but not for *CCND2*.

Table 8: Frequency of positive cases [n(%)] and distribution of methylation levels of cancer-related genes [gene/ACTBx1000:median (IQR⁵)]

Gene	Benign		Malignant		P value
	n (%)	Median	n (%)	Median	
<i>APC</i>	18 (55%)	0.12 (0-1015.4)	144 (81%)	86.85 (0-12878.48)	<0.001
<i>CCND2</i>	22 (67%)	1.30 (0-575357.22)	147 (83%)	86.77 (0-104405.56)	<0.001
<i>RASSF1A</i>	24 (73%)	14.49 (0-1.02E8)	153 (86%)	482.50 (0-31666.68)	<0.001

PERFORMANCE OF METHYLATION MARKERS IN FNA WASHINGS

The diagnostic performance of the three genes was assessed using the cut-off values of methylation levels previously determined for each of these gene promoters (5.0 for *APC*, 2.0 for *CCND2*, and 50.0 for *RASSF1A*) (Jeronimo et al., 2008). ROC curve analysis allowed for the determination for the AUC (CI) for each gene: 0.74 (0.66-0.82) for *APC*, 0.76 (0.68-0.83) for *CCND2*, and 0.72 (0.63-0.81) for *RASSF1A* (Figure 8).

⁵ IQR: Interquartile Range

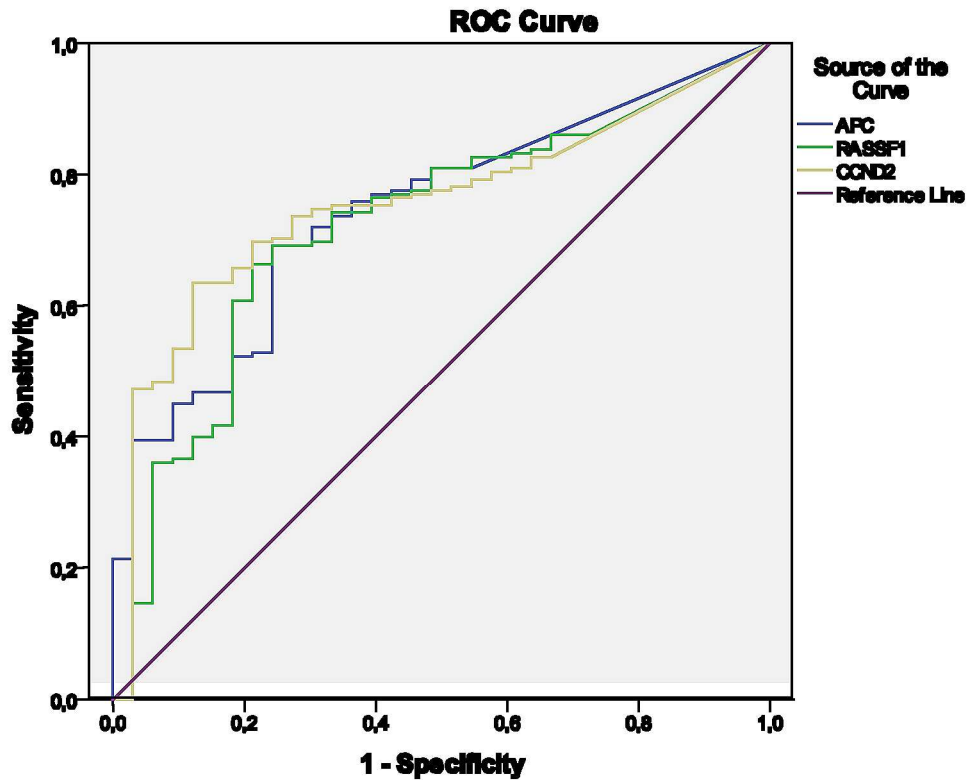


Figure 8: Receiver Operator Characteristic (ROC) curve for each individual gene (*APC*, *RASSF1A* and *CCND2*) in FNA washings from breast lesions

Validity and information estimates considering one, two or three positive markers are displayed in Table 9. The best balance between sensitivity and specificity seems to be obtained with two positive markers (0.78 and 0.79, respectively).

Table 9: Validity estimate for increasing number of positive tested markers in FNA washings from breast lesions

Validity estimates	Number of markers with positive result in each case		
	1	2	3
Sensitivity (95% CI)	0.88 (0.82-0.92)	0.78 (0.71-0.83)	0.37 (0.30-0.44)
Specificity (95% CI) ⁶	0.42 (0.24-0.62)	0.79 (0.62-0.89)	0.91 (0.76-0.97)
Positive LR ⁶ (95% CI)	1.50 (1.078-2.12)	3.65 (1.88-7.09)	4.08 (1.37-12.20)
Negative LR (95% CI)	0.29 (0.17-0.54)	0.28 (0.21-0.39)	0.69 (0.59-0.81)

⁶ CI: Confidence Interval; LR: Likelihood Ratio

CORRELATIONS BETWEEN EPIGENETIC DATA AND CLINICO-PATHOLOGICAL FEATURES

No significant correlations were found between promoter methylation levels and patients' age, tumor grade or pathological stage. However, statistically significant differences in *RASSF1A* and *CCND2* methylation levels between estrogen receptor positive and estrogen receptor negative breast tumors were observed ($P = 0.003$ and $P < 0.001$, respectively). Concerning progesterone receptor status, a significant difference was only observed for *CCND2* methylation levels ($P = 0.011$).

SURVIVAL ANALYSES

The median follow-up of this series of breast cancer patients ($n = 178$) was 57.7 months (range: 0.5 to 90 months). Thirteen (7.3%) patients were lost to follow-up. For the purposes of survival analyses, all cases were coded based on gene methylation levels using as a threshold the value of percentile 75 for each gene. Moreover, grades 1 and 2 were coupled in the same category, against grade 3.

A total of 19 patients (10.7 %) died from breast cancer during the follow-up period. Among all clinical, pathological, and molecular variables analyzed, increased pathological stage, tumor grade, and high-methylation levels of *RASSF1A* were associated with worse overall survival in univariate analysis ($P < 0.001$, $P = 0.018$, and $P = 0.040$, respectively). Disease-specific survival curves using established clinical and pathological variables showed that advanced pathological stage and tumor grade were significantly associated with a worse outcome ($P < 0.001$ for both) (Figures 9 and 10), whereas age, hormone receptor status and gene methylation levels did not show prognostic value within the available follow-up time.

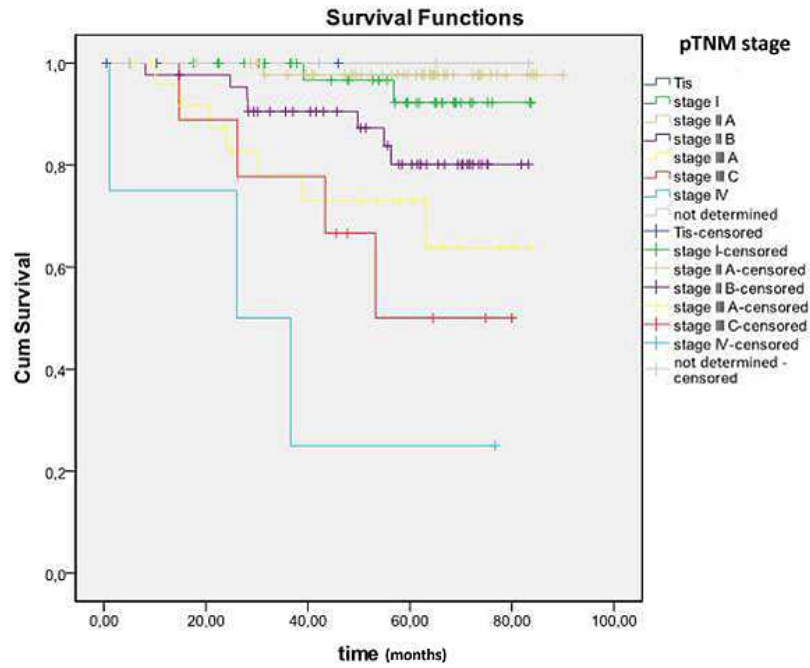


Figure 9: Disease specific survival curve based on pathological stage

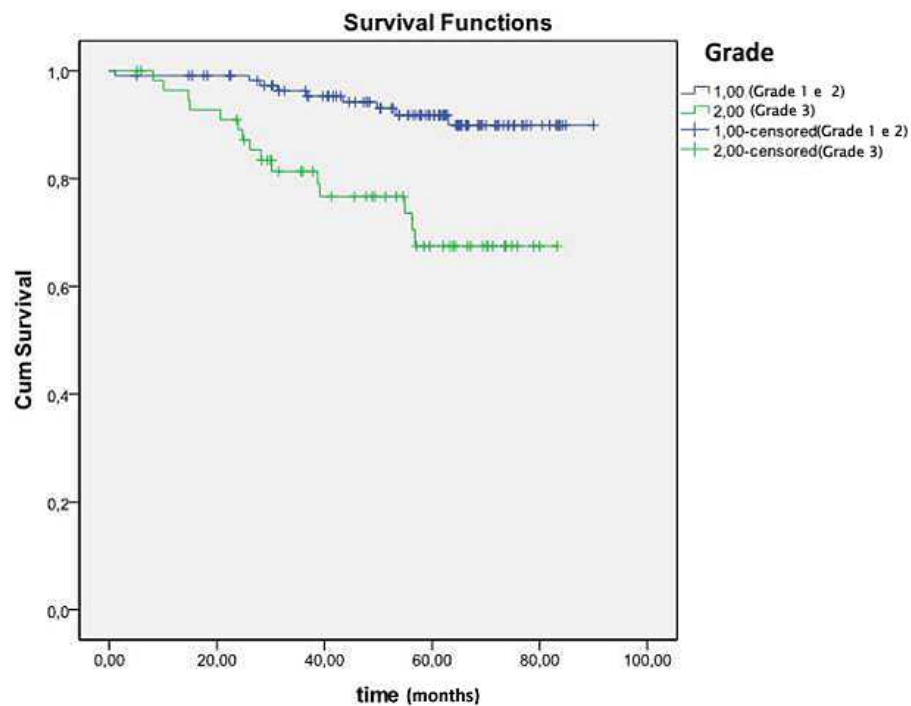


Figure 10: Disease specific survival curve based on tumor grade

Tumor recurrence was detected in 32 (18.0%) patients during the follow-up period. Advanced clinical stage, increased tumor grade, and high-methylation levels of *RASSF1A* (Figure 11) were significantly associated with disease relapse in univariate analysis ($P < 0.001$, $P < 0.001$, and $P = 0.004$, respectively).

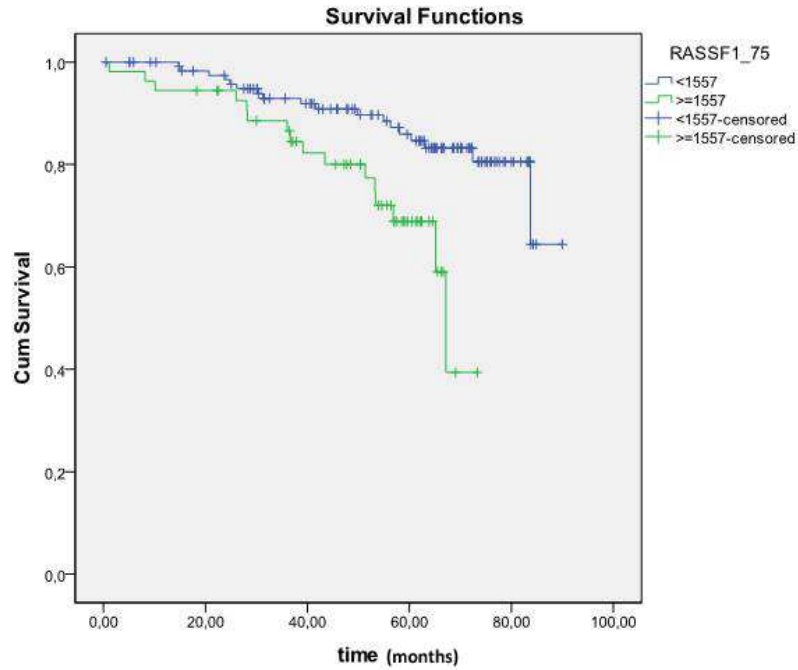


Figure 11: Disease free survival curve based on RASSF1A high-methylation levels

When clinical and epigenetic variables were introduced in a Cox-regression model for the prediction of relapse, pathological stage, tumor grade, and *RASSF1A* methylation levels were selected in the final step of the model as independent predictors (Table 10).

Table 10: Cox regression models assessing the potential of clinical and epigenetic variables in the prediction of overall survival, disease-specific or disease-free survival for 178 breast cancer patients

Model Tested	Variables	Odds Ratio (OR)	95% CI ⁷ for OR	p
Overall Survival	pTNM	1.37	1.12-1.67	0.002
Disease-specific Survival	pTNM	1.51	1.19-1.92	<0.001
	Grade	3.71	1.45-9.51	0.006
Disease-free Survival	pTNM	1.46	1.16-1.80	0.001
	Grade	3.26	1.42-7.50	0.005
	<i>RASSF1A</i> methylation ≥ p75	2.53	1.09-5.87	0.031

⁷ CI: Confidence Interval

DISCUSSION

Cytological evaluation of suspicious breast lesions has been widely performed as an initial triaging procedure to identify malignant lesions and assist the clinician in setting the best strategy to obtain a definitive diagnosis and subsequent therapeutic decisions. However, cytomorphological assessment of breast FNA biopsy specimens meets with important limitations ranging from the cytopathologist's proficiency to the availability of representative material to render a definitive diagnosis. In previous studies, we demonstrated that FNA washings from suspicious breast lesions yield significant amounts of genomic DNA for methylation studies (Jeronimo et al., 2003) and we confirmed the power of a small panel of methylation markers to identify malignant breast cells even in cases with low yield of cytological material, thus providing a valuable ancillary tool to routine cytomorphological observation (Jeronimo et al., 2008). In this study, we extended the spectrum of analysis of epigenetic markers in breast cancer, assessing the prognostic value of quantitative gene promoter methylation in a large series of breast cancer patients.

Overall, the population on which this study is based reflects the referral condition of a cancer institute. Indeed, benign lesions are less than 20% of all cases analyzed as most patients had been already triaged by the respective general physician based on clinical and imagiological information. Thus, most cases were highly suspicious of cancer and that condition was confirmed by FNA biopsy at the cancer institute in the vast majority of cases. This finding highlights the usefulness of the FNA biopsy procedure, although in 22% (52 out of 237) of cases no definitive diagnosis was rendered based on cytomorphological evaluation.

The present series includes 123 of the cases previously reported by our research group (Jeronimo et al., 2008) and it was extended with new consecutive cases, almost doubling the series. This larger series of patients allowed us to perform a confirmatory test of the diagnostic performance of the small panel of methylation markers previously reported to augment the accuracy of FNA biopsy of breast lesions (Jeronimo et al., 2008). However, of the initial panel of four genes, only three loci were analyzed (*APC*, *CCND2*, and *RASSF1A*) owing to the scarcity of DNA available for each sample. Importantly, from our previous findings we concluded that two or three methylation markers would provide adequate ancillary information for breast cancer diagnosis in FNA biopsies (Jeronimo et al., 2008). In the present series, ROC curve analysis confirmed our

previous results concerning the diagnostic performance of individual methylation markers. Interestingly, the validity estimates indicate that the best balance between sensitivity and specificity (around 80% for both) was obtained when two positive markers were used to identify a malignant lesion. It is noteworthy that we used the same cut-off values for gene methylation levels previously determined (Jeronimo et al., 2008), a feature that provides additional validity to the present results.

The cancer specificity of our three gene panel is well demonstrated in the present study as the median levels of methylation at *APC*, *CCND2*, and *RASSF1A* promoters differed significantly between cancerous and non-cancerous samples, confirming our previous observations (Jeronimo et al., 2008). Importantly, these results are in accordance with the findings of other researchers. Pu and co-workers reported on the ability of *RAR β* , *RASSF1A*, and *CCND2* promoter methylation to identify malignancy in FNA samples with indeterminate cytological diagnosis (Pu et al., 2003). Moreover, aberrant methylation in at least one of a three gene panel which included *RASSF1A*, *APC*, and *DAPK1* was positive in 76% of serum samples from breast cancer patients (Dulaimi et al., 2004). These studies confirm the usefulness of epigenetic markers for early and accurate detection of breast cancer, in parallel with similar findings from our research group and others concerning prostate cancer (Henrique et al., 2007a).

However, the main novelty of this study lies on the assessment of the prognostic value of methylation markers quantitatively determined in FNA washings from breast lesions. Indeed, to the best of our knowledge, this is the first study to demonstrate that high-methylation levels of the *RASSF1A* promoter (> p75) assessed in FNA washings is an independent predictor of poor outcome in breast cancer patients. The cut-off value (p75) was based in our previous studies in prostate cancer which demonstrated that high-methylation levels of *APC* were independent predictors of poor outcome (Henrique et al., 2007b). These findings are suggestive of cumulative effect of promoter methylation required to achieve effective gene silencing. Remarkably, *RASSF1A* promoter methylation has been previously identified as a potential prognostic marker for breast cancer in different types of clinical samples. Indeed, Muller and co-workers reported that *RASSF1A* promoter methylation detected in sera or plasma from patients with primary or metastatic breast cancer was associated with poor outcome (Muller et al., 2003). Following the same line of evidence, Hoque and co-workers found that

RASSF1A promoter methylation was more frequent in advanced stage breast cancer patients (Hoque et al., 2006). Interestingly, *RASSF1A* promoter methylation seems to be one of the earliest epigenetic alterations in breast carcinogenesis as it has been found even in benign, atypical breast lesions and carcinoma in situ (Lehmann et al., 2002). Thus, it would be tempting to speculate whether those lesions with higher *RASSF1A* methylation levels would be more prone to progress to invasive cancer.

The only clinicopathological parameters that surfaced as independent predictors of outcome in the present series were pathological stage and tumor grade, whereas hormone receptor status did not. This was a somewhat unexpected result as the expression of estrogen and/or progesterone receptor is associated with favorable prognosis and is highly predictive of response to endocrine treatment (Bardou et al., 2003). Because no selection bias was apparent in our series, this lack of prognostic value for hormone receptor status might be due to insufficient follow-up time. We also did not assess HER2 status in the present series as a significant number of cases were collected prior to the implementation of routine HER2 assessment in breast cancer and, thus, that information was not available in many cases. Nonetheless, it is noteworthy that a molecular assay (quantitative *RASSF1A* promoter methylation) performed in an exiguous sample of cancer cells obtained by FNA was able not only to discriminate malignant from benign lesions, but also to convey relevant prognostic information when compared with standard parameters which require extensive tissue sampling and expert observation.

CONCLUSIONS

In this study, we demonstrated that quantitative assessment of the promoter methylation of three cancer-related genes (*APC*, *CCND2*, and *RASSF1A*) in FNA washings from suspicious breast lesions was able to discriminate malignant from benign breast lesions, augmenting the diagnostic performance of cytopathology. Furthermore, high-methylation level of a single gene (*RASSF1A*) was shown to be an independent predictor of worse outcome in breast cancer. These results support a role for the use of epigenetic markers as ancillary tools in the clinical and pathological assessment of breast cancer patients, requiring validation in larger and independent series. Further studies addressing the development of predictive models for pre-operative staging and therapy response based on epigenetic biomarkers might also provide valuable tools for breast cancer patient management.

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