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Signalling Pathways in Basal-like Breast Carcinomas: A Clue on Pathogenesis and Therapeutical Targets

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Tese de Doutoramento

Programa Doutoral em Biomedicina

Trabalho efectuado sob a orientação de:

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2010

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Financial Support:

PhD fellowship: SFRH / BD / 21551 / 2005

Fundação para Ciência e Tecnologia (FCT)



Para o *Filipe*

“If we knew what it was we were doing, it would
not be called research, would it?”

Albert Einstein

Agradecimentos/Acknowledgements

Ao Prof Fernando Schmitt – Acho que qualquer coisa que pudesse escrever aqui seria sempre pouco para descrever o meu sentimento de gratidão. Durante 8 anos tive o privilégio de trabalhar consigo. Parece uma vida... bem... é uma vida. Espero que não ache um exagero mas os agradecimentos de uma tese são das poucas coisas que são só nossas, neste caso minhas. Depois de olhar para todos estes anos de risos, choros, alegrias, tristezas, discussões e reconciliações... acho que gosto de olhar para si com um carinho que se olha para um Pai. Um Pai científico? Talvez... mas não só. Isso é pouco, muito pouco. Obrigada por tudo.

To Prof Yosef Yarden - I would like to thank the opportunity to be a part of a new scientific world. It was an honor to collaborate with you and your group and being a part of amazing scientific projects. It was wonderful to discover that great scientists are capable of being great men: true models of right conduct and humbleness.

Ao Prof. Sobrinho-Simões por me deixar fazer parte “das suas gentes”. Por tudo o que tem feito pela Ciência em Portugal, e por um dia ter tido um sonho: o IPATIMUP.

À Prof. Raquel Seruca, chefe do GRANDE grupo Cancer Genetics. Pelo espírito crítico e pelos abraços apertadinhos dados nas horas complicadas.

À Fernanda Milanezi e ao José Luís Costa que, para mim, serão sempre co-autores desta tese... mas vamos por partes:

Fê – muito obrigada por tudo, amiga. Pelos milhares de milhões de lâminas que vimos juntas, pelas aulas de patologia que me foste dando, pelo espírito crítico que sempre tiveste e que me ensinou muito sobre rigor científico. Por nunca desistires de mim. Por estares sempre, sempre, sempre presente quando mais precisei. Pela dor que sei que sentiste comigo (e por mim) e por fazeres todo o possível (e o impossível) por me ajudares nas horas mais negras. Tens e terás sempre um lugar no meu coração.

Zé Luís – ou devo dizer o meu “Obi Wan Kenobi” por este percurso científico alucinante. Contigo aprendi a aprender. Sabes que “o portuguesinho” não gosta de ser criticado. Eu, claro também não era excepção. Graças a ti acho que passei a ser. Sem dúvida nenhuma que com a crítica se aprende muito mais. E as tuas críticas foram sempre didácticas e só tenho que te agradecer por isso. Tivemos e quem sabe se no futuro não continuaremos (espero que sim) a ter “brainstorms” científicos de *altíssimo gabarito* e foi muito bom poder discutir o meu trabalho (e um sem número

de outras ideias) contigo. Em termos de laboratório, foste provavelmente o “professor” mais rigoroso que tive. Mas sem dúvida alguma com quem aprendi mais.

À minha amiga Sara Ricardo. Por estares sempre lá. Nas horas boas e nas horas más. Pelas risadas, conversas, partilhas de vida...

Aos restantes elementos do grupo da Mama: Joana, André, Ana Sofia, Babi, Nair, Madalena, Diana e o André Vieira. E a todos os restantes Cancer Genetics.

Aos meus amigos do coração (e da barriga): Joana Figueiredo, Ana Sofia Ribeiro, Joana Carvalho, Hugo Seca, Rui Ferreira pelos anos de almoços de chorar a rir. Pelas vivências que partilhamos... e pela certeza que - Vou sentir muito a vossa falta onde quer que esteja.

To my dear friends at the Weizmann, particularly Nir, Fresia, Hadas, Gur, Gabi, Daniela and Mattia. It's amazing how 3 months can change a life. Thank you, for making me feel at home. To all the department of Biological Regulation. To Sara for being our lab mom. A special thanks to Lesley and Marti, two of the sweetest persons I have ever knew. To all the good friends I made at the Clore House.

Ao Paulo Canedo, Zé Luís e Pati Oliveira pelas risadas que demos juntos, pelos bons momentos que partilhamos e por fazerem com que a escrita da minha tese não fosse o fim do mundo.

À Patrícia Oliveira, pelos gritos de alma (entenda-se risadas) e por seres tão fixe.

À Cristina Madureira, pelo incentivo constante, e claro como não podia deixar de ser pelas risadas.

Aos meninos do gabinete por me aturarem. Eu sei, só faço barulho.

À Zézinha, à Cátia, à D. Alcina, ao Sr. Oliveira e ao Sr. Mendes, ao Sr. Mário, à Fátima Pinto, à Isabel, à Manuela um beijinho muito grande.

Aos meus pais, pelo incentivo e pelo apoio desde sempre. Pelo carinho e por me aturarem... eu sei que tenho mau feitio... por estarem sempre presentes e pela certeza que tenho (e que é bom ter) que com eles tenho sempre um porto seguro.

À minha mãe... é um facto, Mãe só há mesmo uma... e a minha é das boas.

Ao meu irmão, à minha cunhada que nunca, nunca, nunca me deixaram à deriva e sempre foram acima de tudo meus amigos. É bom saber que aconteça o que acontecer posso sempre contar convosco.

À minha avó Ju. A minha avó Ju.... A minha avó Ju. A vida da minha avó dava um livro (mas dos bons). Mesmo com uma vida nem sempre fácil, a minha avó foi, é e será sempre um exemplo de vida. A minha avó é um Doce. Espero um dia ter filhos ou netos que tenham por mim 1/10 da admiração, carinho e amor que eu tenho por ela. Obrigada avó.

E por fim ao Filipe, a quem dedico esta tese. Infelizmente, este foi um caminho que não pudemos fazer juntos. Mas sei que terias o maior orgulho em mim. Contigo aprendi tantas coisas... e tantas sei que ficaram por aprender. Faz-me falta o meu companheiro, mas esse mesmo companheiro deu-me a alegria de viver. E esse é um compromisso que vou honrar.

Ao abrigo do Art. 8º do Decreto-Lei n.º388/70 fazem parte integrante desta dissertação os seguintes trabalhos já publicados:

I - SILVA F, **CARVALHO S**, MILANEZI F, SCHMITT FC (2008) BASAL-LIKE CARCINOMA OF THE BREAST. ACTA MED PORT 21: 373-8

II - SOUSA B, PAREDES J, MILANEZI F, LOPES N, MARTINS D, DUFLOTH R, VIEIRA D, ALBERGARIA A, VERONESE L, CARNEIRO V, **CARVALHO S**, COSTA JL, ZEFERINO L, SCHMITT F (2010) P-CADHERIN, VIMENTIN AND CK14 FOR IDENTIFICATION OF BASAL-LIKE PHENOTYPE IN BREAST CARCINOMAS: AN IMMUNOHISTOCHEMICAL STUDY. HISTOL HISTOPATHOL 25: 963-974

III - REIS-FILHO JS, MILANEZI F, **CARVALHO S**, SIMPSON PT, STEELE D, SAVAGE K, LAMBROS MB, PEREIRA EM, NESLAND JM, LAKHANI SR, SCHMITT FC (2005) METAPLASTIC BREAST CARCINOMAS EXHIBIT EGFR, BUT NOT HER2, GENE AMPLIFICATION AND OVEREXPRESSION: IMMUNOHISTOCHEMICAL AND CHROMOGENIC IN SITU HYBRIDIZATION ANALYSIS. BREAST CANCER RES 7: R1028-35

IV - REIS-FILHO JS, PINHEIRO C, LAMBROS MB, MILANEZI F, **CARVALHO S**, SAVAGE K, SIMPSON PT, JONES C, SWIFT S, MACKAY A, REIS RM, HORNICK JL, PEREIRA EM, BALTAZAR F, FLETCHER CD, ASHWORTH A, LAKHANI SR, SCHMITT FC (2006) EGFR AMPLIFICATION AND LACK OF ACTIVATING MUTATIONS IN METAPLASTIC BREAST CARCINOMAS. J PATHOL 209: 445-53

V - MILANEZI F, **CARVALHO S**, SCHMITT FC (2008) EGFR/HER2 IN BREAST CANCER: A BIOLOGICAL APPROACH FOR MOLECULAR DIAGNOSIS AND THERAPY. EXPERT REV MOL DIAGN 8: 417-34

VI – **CARVALHO S**, MILANEZI F, COSTA JL, AMENDOEIRA I, SCHMITT F (2010) PIKING THE RIGHT ISOFORM: THE EMERGENT ROLE OF THE P110BETA SUBUNIT IN BREAST CANCER. VIRCHOWS ARCH 456: 235-43

VII – **CARVALHO S**, SCHMITT F (2010) THE POTENTIAL ROLE OF PI3K INHIBITORS IN THE TREATMENT OF BREAST CANCER. FUTURE ONCOLOGY [IN PRESS]

VIII - KATZ M, AMIT I, CITRI A, SHAY T, **CARVALHO S**, LAVI S, MILANEZI F, LYASS L, AMARIGLIO N, JACOB-HIRSCH J, BEN-CHETRIT N, TARCIC G, LINDZEN M, AVRAHAM R, LIAO YC, TRUSK P, LYASS A, RECHAVI G, SPECTOR NL, LO SH, SCHMITT F, BACUS SS, YARDEN Y (2007) A RECIPROCAL TENSIN-3-CTEN SWITCH MEDIATES EGF-DRIVEN MAMMARY CELL MIGRATION. NAT CELL BIOL 9: 961-9

IX - MOSESSON Y, CHETRIT D, SCHLEY L, BERGHOFF J, ZIV T, **CARVALHO S**, MILANEZI F, ADMON A, SCHMITT F, EHRLICH M, YARDEN Y (2009) MONOUBQUITINYLATION REGULATES ENDOSOMAL LOCALIZATION OF LST2, A NEGATIVE REGULATOR OF EGF RECEPTOR SIGNALING. DEV CELL 16: 687-98

NOTA EXPLICATIVA:

A presente dissertação encontra-se maioritariamente escrita em inglês uma vez que o Professor Yosef Yarden do Department of Biological Regulation do Weizmann Institute of Science em Rehovot, Israel ter sido co-orientador.

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ABBREVIATIONS

ASCO – **A**merican **S**ociety of **C**linical **O**ncology
AKT – v-akt murine thymoma viral oncogene homolog
APC - **A**denomatous **P**olyposis **C**oli
ATP – **A**denosine **t**riphosphate
AUS – **A**ntigen **U**nmasking **S**olution
BRAF – v-raf murine sarcoma viral oncogene homolog B1
BRCA1 – **B**reast **C**ancer **1**, early onset
BRCA2 – **B**reast **C**ancer **2**, early onset
c-FOS – (alias for FOS) FBJ murine osteosarcoma viral oncogene homolog
c-KIT – v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CISH – **C**hromogenic *in situ* **H**ybridization
CK - **C**ytokeratin
CTEN – **C**-terminal **T**ensin-like Protein
DEG – **D**elayed **E**arly **G**ene
DEP1 – **D**ensity **E**nhanced **P**hosphatase **1**
DFS – **D**isease **F**ree **S**urvival
DSB – **D**ouble **S**trand **B**reak
ECD – **E**xtracellular **D**omain
EDTA – **E**thylenediaminetetracetic **A**cid
EGF – **E**pidermal **G**rowth **F**actor
EGFR – **E**pidermal **G**rowth **F**actor **R**eceptor
ELK1 – ELK1, member of ETS oncogene family
EMA – **E**pithelial **M**embrane **A**ntigen
ER – **E**strogen **R**eceptor
ERK – **E**xtracellular signal **R**egulated **K**inase
FDA – **F**ood and **D**rug **A**dministration
GDP – **G**uanidine **D**iphosphate
GEF – **G**uanine-nucleotide **E**xchange **F**actor
GEP – **G**ene **E**xpression **P**rofile
GPCR – **G** **P**rotein-**C**oupled **R**eceptor
GRB2 – **G**rowth **F**actor **R**eceptor **B**ound Protein **2**
GRB7 – **G**rowth **F**actor **R**eceptor **B**ound Protein **7**

GTP – **G**uanidine **T**riphosphate
H&E – **H**aematoxinilin and **E**osin
HER2 – **H**uman **E**pidermal **R**eceptor 2
HR – **H**ormonal **R**eceptors
hLST2 – **L**ateral **S**ignaling **T**arget Protein 2 **H**omolog
HSP – **H**eat **S**hock **P**rotein
IDC-NOS – **I**nvasive **D**uctal **C**arcinoma **N**ot **O**therwise **S**pecified
ID4 - Inhibitor of **D**N**A** binding **4**
IR – **I**nsulin **R**eceptor
LN – **L**ymph **N**ode
LST2 - **L**ateral **S**ignaling **T**arget Protein 2
mAb – **M**onoclonal **A**ntibody
MAPK – **M**itogen-activated **P**rotein **K**inase
MEK – (alias for MAP2K) Mitogen Activated Protein Kinase Kinase
MM – **M**ouse **M**onoclonal
MNK1 – (alias for MKNK1) MAP Kinase interacting serine/threonine kinase 1
MSH3 - **MutS** Homolog **3**
mTOR – **M**ammalian **T**arget of **R**apamycin
MYC – v-myc myelocytomatosis viral oncogene homolog
NFκB – **N**uclear **F**actor of **K**appa light polypeptide enhancer in **B** cells
NGFR – **N**erve **G**rowth **F**actor **R**eceptor
NOS – **N**ot **O**therwise **S**pecified
NRG – **N**euregulin
NSCLC – **N**on **S**mall **C**ell **L**ung **C**arcinoma
ON - **O**vernight
OS – **O**verall **S**urvival
PARP – **P**oly **A**D**P**ribose **P**olymerase
PCR – **P**olymerase **C**hain **R**eaction
PDK1 – **P**yruvate **D**ehydrogenase **K**inase, isozyme **1**
PI - **P**hosphatidylinositol
PI3K – **P**hosphatino**s**itide-**3**-**K**inase
PIK3CA – **P**hosphoinositide-**3**-**K**inase, catalytic, **a**lpha polypeptide
PIK3CB – **P**hosphoinositide-**3**-**K**inase, catalytic, **b**eta polypeptide
PIP₂ – **P**hosphatydilinositol-4,5-bisphosphate

PIP₃ - **Phosphatidylinositol-3,4,5-trisphosphate**

PR – **Progesterone Receptor**

PTEN – **Phosphatase and Tensin** homolog

PTP1B – **Protein Tyrosine Phosphatase 1B**

QRT-PCR - **Quantitative Reverse Transcriptase Polymerase Chain Reaction**

RAD17 – RAD17 homolog

RAD50 – RAD50 homolog

RAS – v-ras rat sarcoma viral oncogene homolog (K – Kirsten; H – Harvey; N – Neuroblastoma)

RM – **Rabbit Monoclonal**

RTK – **Receptor Tyrosine Kinase**

siRNA – **Short Interfering RNA**

SM – **Smooth Muscle**

SMA – **Smooth Muscle Actin**

SOCS5 – **Suppressor of Cytokine Signalling 5**

SOS – **Son of Sevenless** homolog

SRC – v-src sarcoma (Shmidt – Rupp A2) viral oncogene homolog (avian)

STAT3 - **Signal Transducer and Activator of Transcription 3**

SYNJ2 – **Synaptojanin 2**

S6K – (alias of RPS6KB1) Ribosomal protein **S6 Kinase**

TDLU – **Terminal Ductal Lobular Unit**

TGFα – **Transforming Growth Factor alpha**

TKI – **Tyrosine Kinase Inhibitor**

TMA – **Tissue Microarray**

TNS – **Tensin**

TP53 – **Tumour Protein p53**

WHO – **World Health Organization**

XRCC4 - **X-RAY REPAIR COMPLEMENTING DEFECTIVE REPAIR IN CHINESE HAMSTER CELLS 4**

ABSTRACT

Breast cancer is a complex and heterogeneous disease, comprising multiple entities associated with distinct histological patterns, biological features and clinical behaviours. The traditional pathological classification is considered suboptimal, since patients with identical tumour types and stage of disease can have markedly contrasting outcomes. Recent gene expression profiling of breast cancer has identified specific subtypes with clinical, biologic, and therapeutic implications. These can be divided into: luminal A, luminal B, HER2-overexpressing and basal-like breast carcinomas. This latter group, has captured the attention of oncologists and scientists since no specific therapeutic regimen is currently available for their treatment. The studies described in the present thesis aimed at improving our understanding on the underlying mechanisms of basal-like breast carcinomas development/progression. **Chapter 1** comprises the construction of an invasive breast carcinomas tissue bank and its classification into the different subtypes of breast carcinomas. Basal-like breast carcinomas corresponded to 9.2% of the cases. They were of higher grade, presented a higher proliferation rate, were more frequently associated with the development of locoregional and distant metastasis and presented worse overall survival. As still no consensual immunohistochemical panel for the definition of basal-like breast carcinomas is accepted, other markers like P-cadherin, CK14 and Vimentin were also tested, as all have been previously associated with basal-like phenotype. All three markers were able to identify basal-like breast carcinomas particularly when associated with CK5. Furthermore P-cadherin was able to recognize possible basal-like tumours among triple negative tumours that do not express CK5 or EGFR. Several potential targets, namely EGFR, have emerged from this search for specific markers. As anti-EGFR targeted therapies are already used in the clinical setting for the treatment of other tumors, like lung cancer, in **Chapter 2** we determined the importance of EGFR in basal-like breast carcinomas. For that we used the invasive breast carcinoma series and also a specific subgroup of breast carcinomas previously associated with basal-like features – the metaplastic breast carcinomas. EGFR was overexpressed in 5.7% of invasive breast carcinomas and in 68% of metaplastic breast carcinomas. Next we determined possible mechanisms that could account for the high EGFR expression observed in metaplastic breast carcinomas. By chromogenic *in situ* hybridization we observed that 34% of the cases presented gene amplification. As most cases that presented overexpression did not have gene amplification we also searched for the presence of activating mutations. None were found. As EGFR and other basal-like associated proteins like c-KIT, $\alpha 6\beta 4$ integrin or α -crystallin are known to activate both PI3K and RAS-RAF-MAK in **Chapter 3** we sought to determine the involvement

of these signalling pathways in the development/progression of basal-like breast carcinomas. We assessed several downstream effectors of these pathways as well as the mutational status of *PIK3CA*. p110 α was more frequent in hormone receptor positive cases, but when considering the different subtypes it was mostly expressed in luminal A and basal-like tumours. However its expression was not associated with presence of mutations in *PIK3CA*. Our series presented a 19.2% mutation frequency for *PIK3CA* and the mutations occurred mainly in exon 20. p110 β was highly associated with HER2-overexpressing tumours. Activation of AKT was observed in the majority of the analysed cases irrespective of their subtype. The most striking observation in the analysed effectors was the lack of PTEN expression in basal-like breast carcinomas. This absence of expression occurred in 64.5% of the cases. Regarding p-ERK1/ERK2 it was possible to observe that this is not a preferential pathway in basal-like breast carcinomas. As breast cancer is able to make use of many signalling cues in **Chapter 4** we refined the knowledge on EGFR network dynamics. The EGF-induced, C-terminal tensin like domain (CTEN) and Synaptojanin2 (SYNJ2), were initially identified in the analysis of a data set that used EGFR as a model system to study signalling networks. The human lateral signaling target protein 2 (LST2), was studied in the context of negative regulation of EGFR. In the invasive breast carcinoma series, CTEN expression was associated with the expression of both EGFR and HER2 and was inversely correlated with ER. Furthermore, CTEN expressing tumours presented higher histological grade and frequently metastasized to lymph nodes. CTEN positivity was mostly observed in basal-like carcinomas. SYNJ2 was also more frequently observed in basal-like and HER2-overexpressing tumours, which is in agreement with its attributed role in a more aggressive phenotype. ER negative tumours were more frequently SYNJ2 positive and although not statistically significant SYNJ2 positive tumours presented more frequently distant metastasis. LST2, a negative regulator of EGFR signalling, was expressed in 4.4% of cases mainly luminal A. Taken together our results, suggest the use of a panel of immunohistochemical markers for the identification of basal-like breast carcinomas. Furthermore we provide evidence for the association of EGFR with basal-like breast carcinomas particularly in the subset of metaplastic breast carcinomas. This association provides proof of principle for the treatment with anti-EGFR drugs. Nonetheless predictive biomarkers of response, namely PTEN need to be taken into consideration in order to achieve the expected results. Also systems biology approaches will enable the identification and prediction of signalling outcomes that can be translated into the clinic.

RESUMO

O cancro da mama é uma doença complexa e heterogénea, que engloba várias entidades, associadas com padrões histológicos específicos, características biológicas e comportamentos clínicos. A classificação morfológica tradicional é no entanto considerada sub-ótima uma vez que, pacientes com o mesmo tipo e grau histológico podem apresentar prognósticos diversos bem como diferentes respostas à terapia. Recentemente, os perfis de expressão génica identificaram subtipos específicos de cancro da mama com implicações clínicas, biológicas e terapêuticas. Estes, podem ser classificados em: luminal A, luminal B, com sobre-expressão de HER2 ou do tipo basal. Este último grupo, tem sido particularmente estudado uma vez que apresenta um comportamento agressivo e actualmente não tem um tratamento específico. O trabalho descrito na presente tese teve como objectivo a identificação de moléculas alvo que poderiam ser importantes para o desenvolvimento e progressão do carcinoma da mama do tipo basal. Assim, o **Capítulo 1** consistiu na construção de um banco de tumores composto por carcinomas invasivos da mama que foram posteriormente classificados nos diferentes subtipos. Os carcinomas da mama do tipo basal representavam 9,2% da série. Eram de alto grau, com um alto índice proliferativo, frequentemente apresentavam metastização locoregional ou à distância bem como pior sobrevida. Uma vez que actualmente não existe um painel consensual de marcadores para a identificação de carcinomas basais, testamos os marcadores P-caderina, CK14 e Vimentina (previamente associados com o fenótipo basal) para determinar a sua sensibilidade e especificidade. Todos os marcadores foram capazes de identificar os carcinomas basais particularmente quando associados à CK5. Para além disso, a P-caderina identificou possíveis carcinomas da mama do tipo basal entre os carcinomas triplo negativos sem expressão de CK5 ou EGFR. Nestes estudos de diferentes marcadores, alguns potenciais alvos terapêuticos, como por exemplo o EGFR, foram identificados. Uma vez que as terapias anti-EGFR já são utilizadas na clínica para o tratamento de tumores, como por exemplo do pulmão, no **Capítulo 2** fomos determinar a importância do EGFR nos carcinomas da mama do tipo basal. Para isso utilizamos a série de carcinomas invasivos da mama previamente descrita bem como um subgrupo específico de tumores – os carcinomas metaplásicos da mama. O EGFR encontrava-se sobre-expresso em 5,7% dos carcinomas invasivos da mama e em 68% dos carcinomas metaplásicos da mama. Com base nestes resultados fomos determinar, nos carcinomas metaplásicos da mama, possíveis mecanismos responsáveis por esta sobre-expressão. Por hibridização *in situ* por cromogénio observamos que em 34% dos casos sobre-expressos ocorria amplificação génica. Uma vez que este mecanismo não era responsável pelos

restantes casos de sobre-expressão, pesquisamos mutações ativadoras do receptor que não foram detectadas. Não só o EGFR mas outros genes associados com o fenótipo basal como o c-KIT, a $\alpha 6\beta 4$ integrina ou a α -cristalina, são capazes de activar quer a via da PI3K quer a via RAS-RAF-MAPK pelo que, no **Capítulo 3** o envolvimento destas vias de sinalização no desenvolvimento/progressão dos carcinomas de mama do tipo basal foi estudado. Vários efetores das vias bem como o status mutacional do gene *PIK3CA* foram analisados. A expressão de p110 α era mais frequente nos casos positivos para receptores hormonais, no entanto quando avaliada tendo em conta a classificação molecular encontrava-se mais expressa nos carcinomas luminal A e do tipo basal. Contudo esta expressão não se encontrava associada com a presença de mutações ativadoras no seu gene codificante - *PIK3CA*. A nossa série apresentava uma frequência mutacional para o *PIK3CA* de 19,2%, sendo as mutações mais frequentes no exão 20. A proteína p110 β encontrava-se altamente associada com tumores com sobre-expressão de HER2. Por seu lado a activação do AKT foi observada na maioria dos casos independentemente do subtipo analisado. No entanto a observação mais importante foi a ausência de expressão do supressor tumoral PTEN nos carcinomas da mama do tipo basal, que ocorreu em 64,5% dos casos. Verificou-se que a p-ERK1/ERK2 não se encontrava activada nos carcinomas da mama do tipo basal pelo que não parece que esta via seja preponderante para este tipo de tumores. Uma vez que o cancro da mama é capaz de utilizar inúmeras vias, no **Capítulo 4** decidimos refinar o conhecimento sobre a dinâmica de rede do EGFR. Assim, recorrendo a dados provenientes de um estudo desenvolvido sobre redes de sinalização (utilizando o EGFR como modelo) decidimos analisar o papel de dois genes sobre-regulados pelo EGF, CTEN e SYNJ2. Para além destes, a proteína LST2 foi também analisada mas no seu papel de regulador negativo da via do EGFR. Na série de carcinomas invasivos da mama, a expressão de CTEN encontrava-se associada com a sobre-expressão de EGFR e HER2 e estava inversamente relacionada com a expressão do receptor de estrogénio. Os tumores com sobre-expressão de CTEN eram de alto grau e frequentemente metastizavam para os gânglios linfáticos. Os casos CTEN positivos eram maioritariamente do tipo basal. A expressão de SYNJ2 era mais frequente nos carcinomas da mama do tipo basal e nos com sobre-expressão de HER2, o que corrobora a sua associação com fenótipos mais agressivos. A expressão de SYNJ2 encontrava-se inversamente relacionada com a expressão de receptor de estrogénio. Apesar de não ser estatisticamente significativo verificou-se que os tumores positivos para SYNJ2 apresentavam frequentemente metástases à distância. A LST2, um regulador negativo da sinalização via EGFR, foi expressa em 4,4% dos casos os quais eram na sua maioria luminal A.

Em conclusão, os nossos resultados sugerem a utilização de um painel de marcadores imunohistoquímicos para a identificação de carcinomas da mama do tipo basal. Para além disso demonstramos a associação do EGFR com este subtipo, particularmente nos carcinomas metaplásicos da mama. Esta associação sugere a utilização de terapias anti-EGFR para o tratamento destes tumores. É no entanto, também necessário estudar possíveis marcadores preditivos de resposta a estas terapias, como por exemplo o PTEN. Análises mais integrativas (como as obtidas recorrendo a biologia de sistemas) permitem identificar e prever respostas celulares que poderão ser posteriormente utilizados na clínica.

GENERAL INTRODUCTION

THE MAMMARY GLAND

The mammary gland is composed of a combination of multiple cells that together form complex interaction networks required for the proper development and functioning of the organ [1]. Its development occurs in defined stages that are connected to sexual development and reproduction. Two cellular compartments contribute to the gland: the epithelium and the surrounding stroma, which are derived from ectoderm and mesoderm, respectively [2].

The human breast contains six to ten major ductal systems. The epithelium of the overlying skin dips into the orifices at the nipple and then abruptly changes to a double layered cuboidal epithelium lining the ducts. Branching of the large ducts ultimately leads to the terminal ductal lobular unit (TDLU). In adult women the terminal duct branches into a grape-like cluster of small acini to form a lobule (Figure 1a-c) [3]. The epithelial cells that compose the gland are arranged in two layers, the luminal/epithelial layer and the myoepithelial layer, being the whole structure surrounded by a basement membrane (Figure 1d and e) [4].

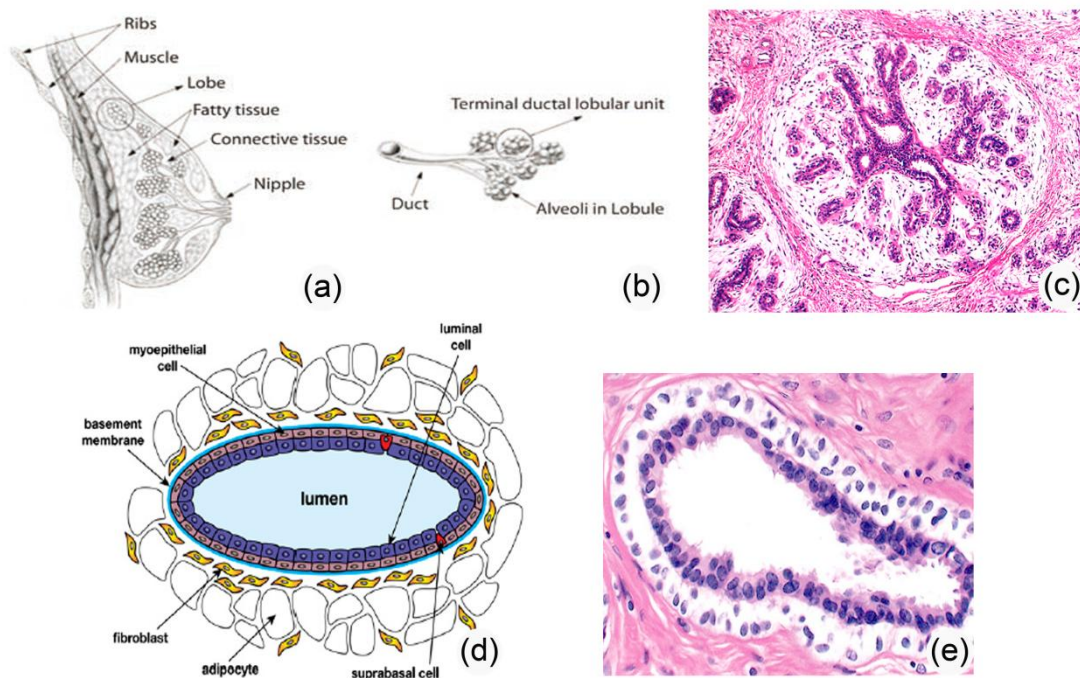


Figure 1 - (a) Schematic representation of the anatomy of the breast; (b) terminal ductal lobular unit (TDLU) location; and (c) Histological picture of a normal mature TDLU, composed of acini surrounded by intralobular connective tissue – H&E 10x (d) Schematic representation of a mammary duct where the different types of cells that constitute it are represented (e) Segmental breast duct: the epithelial and myoepithelial cells can be easily identified separately – H&E 100x (a and b) adapted from www.my-breast-cancer-guide.com/images/breast.gif and (d) adapted from: Visvader JE (2009) Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes Dev* 23: 2563-77

The luminal cells are responsible for milk production, whereas the myoepithelial cells assist in milk ejection during lactation and provide structural support to the lobules. Crosstalk between the mammary epithelium and stroma is also essential for the proper patterning and function of the normal mammary gland [5]. There are two types of breast stroma: the interlobular, which consists of dense fibrous connective tissue, admixed with adipose tissue; and an intralobular stroma that encloses the acini and the lobules and consists of breast-specific hormonally responsive fibroblast-like cells mixed with lymphocytes. In the prepubertal breast of males and females, the large duct system ends in terminal ducts with minimal lobule formation, being the changes in the breast more dynamic and pronounced during the reproductive years. In the females, the development of the mammary gland is tightly regulated by the ovary [6]. In the first half of the menstrual cycle the lobules are relatively quiescent. After ovulation, under the influence of estrogen and rising of progesterone levels, cell proliferation increases, as does the number of acini per lobule, and intralobular stroma becomes markedly edematous. Upon menstruation, the decreasing in the levels of estrogen and progesterone induces regression of the lobules and the disappearance of the stromal edema. Only with the onset of pregnancy the breast becomes completely mature and functional. Lobules increase progressively in number and size. Immediately after the baby's delivery, the luminal cells of the lobules produce colostrum, which then changes to milk. When lactation ends, the breast epithelium and stroma undergo extensive remodelling [7]. The epithelial cells undergo apoptosis, lobules regress and atrophy, and the total breast size is diminished. However, full regression does not occur, and as a result of pregnancy there is a permanent increase in the size and number of lobules. After the third decade, long before menopause, lobules and their specialized stroma start to involute. There is also a change in the interlobular stroma, where the dense fibrous stroma is progressively replaced by adipose tissue [3].

BREAST CANCER – EPIDEMIOLOGICAL, CLINICAL AND HISTOLOGICAL FEATURES

Breast cancer is the most common non-cutaneous malignancy in women, with around one million new cases diagnosed per year [8]. Although breast cancer incidence was commonly associated with Western world [9] a two-fold or three-fold increase in the incidence rates in countries like, Japan, Singapore, China, Korea and more recently India, proved that currently it has to be seen as a global problem [8]. According to the International Agency for Research on Cancer (www.iarc.fr), in Portugal breast cancer is the first leading cause of cancer related deaths amongst women. There are around 4500 new cases per year, with an expectancy of 1 in every

10 women developing the disease throughout life. Since 1994 breast cancer mortality rate has declined [3], with this decrease attributed to early diagnosis, better and more efficient treatment modalities, and a greater awareness and investment in education for early disease detection. Several risk factors are associated with breast cancer development [3]: (a) gender - only 1% of breast cancers occur in men; (b) age - the incidence rises throughout a woman's lifetime (women over 60 years old have a greater risk than younger women); (c) early menarche or late menopause; (d) age at first live birth - women who experience a first full term pregnancy at ages younger than 20 years have half the risk of nulliparous women or women over the age of 35 at their first birth); (e) family history - the risk of breast cancer increases with the number of affected first degree relatives, especially if the cancer occurred at young age; (f) estrogen exposure - it is known that postmenopausal hormone replacement therapy increases the risk of breast cancer (1.2 to 1.7-fold) and adding progesterone increases the risk further; oral contraceptives have not been shown to affect breast cancer risk. Reducing endogenous levels of estrogens by oophorectomy decreases the risk of developing breast cancer by 75%. Alongside with all these risk factors are also, race and ethnicity, breast density and radiation exposure, a history of prior biopsies presenting atypical hyperplasia, and lifestyle (geographic influence, diet, obesity, exercise, breastfeeding and alcohol). When taking into account all the risk factors previously mentioned, breast cancer can be divided into two major groups: sporadic and hereditary, being the first one more related to hormonal exposure, and the second one, with germline mutations, mainly in *Breast Cancer 1, early onset (BRCA1)* and *Breast Cancer 2, early onset (BRCA2)* genes.

The high degree of heterogeneity observed in breast cancer is a by-product of complex genetic and epigenetic changes that drive carcinogenesis. There are commonly accepted traits of tumour development that are formally known as “the hallmarks of cancer” [10], that consist in the acquisition of limitless replicative potential, a capability of sustained angiogenesis, insensitivity to anti-growth signals, self-sufficiency in growth signals, apoptosis evasion and the ability of invading and metastasizing. This concept emphasizes the biological basis of the disease, establishing a conceptual framework for understanding, amongst others, breast cancer heterogeneity.

The vast majority of breast malignancies are adenocarcinomas, which can be divided into two main categories: *in situ* and invasive [3]. *In situ* carcinomas are preinvasive lesions characterized by a proliferation of clonal, neoplastic cells, filling either the ducts or lobules but limited by the

basement membrane. Invasive carcinomas comprise fully malignant neoplasms that have passed through the basement membrane, thus presenting metastatic capabilities.

Historically it was believed that ductal and lobular carcinomas were originated from different cells (for example ductal carcinomas from ductal epithelial cells and lobular carcinomas from the epithelial cells of the terminal ductal lobular units) and would then evolve from distinct morphological precursors in a multistep fashion. It is now accepted that this is not the case. Several lines of evidence suggest that most if not all breast cancers arise from cells in the TDLU [11-13], being the terms ductal and invasive neither related to histogenesis, nor to the site of origin in the mammary gland, but rather with architectural patterns, cytological features and immunohistochemical profiles [14]. Breast cancer can be classified and stratified based upon histological systems according to tumour intrinsic characteristics. The classification most commonly used is the World Health Organization (WHO) [15]. This classification recognizes the existence of 18 types of breast cancer. The large majority (40-75%) are called invasive ductal carcinomas not otherwise specified (IDC-NOS), defined as tumours in which over 50% of their mass displays a non-special pattern. The WHO classification also provides accurate definitions for pure and mixed types of breast cancer, when carcinomas harbour morphological features of one of the recognized entities in more than 90% of the tumour mass or, when it presents 10-49% of ductal NOS pattern and the remaining tumour mass includes a recognized special type, respectively.

Histological type and grade provide complementary information about clinicopathological features of the tumours [16, 17]. Several histological types are associated with favourable prognosis like, medullary, tubular, mucinous, invasive cribriform, amongst others [18]. For grading, the most commonly used system is the Nottingham Histologic Score (also referred as the modified Scarff-Bloom-Richardson scoring system) that assesses the degree of differentiation (tubule/gland formation and nuclear pleomorphism) and proliferative activity (mitotic counts) thus evaluating tumour aggressiveness [15, 19]. It classifies invasive carcinomas into three groups (Grade 1 – well differentiated; Grade 2 – moderately differentiated; Grade 3 – poorly differentiated), that are highly correlated with survival [3]. The size of an invasive carcinoma is associated with the risk of developing axillary lymph node metastasis, that is, the higher the size of a given tumour, the higher the probability of developing lymph node metastasis (even though both parameters are independent prognostic factors) [3]. Axillary lymph node status is the most important prognostic factor, in the absence of distant metastasis. It is well established that, with no nodal involvement, the 10-year disease free survival (DFS) rate is close to 70-80%, whereas when there is one to

three positive nodes it decreases to 35-40% and when more than 10 nodes are compromised the DFS is 10-15% [3]. Hormonal receptors expression is associated with better outcome as these tumours tend to grow slowly, and are better differentiated [20]. It is expected that 80% of tumours expressing both estrogen and progesterone receptors (ER and PR) will respond to hormonal therapy, whereas only 40% of those with either ER or PR alone will. Noteworthy ER positive tumours are less likely to respond to chemotherapy conversely to the negative cases [3]. The human epidermal receptor 2 (HER2) is a receptor tyrosine kinase (RTK), which presents overexpression in 15-30% invasive breast carcinomas [21, 22], mainly associated with gene amplification [23]. Although initially the use of HER2 status as a prognostic marker was controversial, the development of trastuzumab, a humanized monoclonal antibody against HER2 [24], enabled its acceptance in the daily workup of breast cancer management. In the American Society of Clinical Oncology (ASCO) recommendations for the use of tumour markers in breast cancer, HER2 has assumed a relevant position, along with hormone receptor status [25]. The prognostic significance of invasive breast cancers relies on histological type, histological grade, tumour size, presence of lymph node metastasis and lymphovascular invasion assisted by predictive markers such as, hormone receptors, like ER and PR and the human epidermal receptor 2 (HER2) [26]. However, the traditional pathologic classification and staging system is still considered to be suboptimal, as patients with identical tumour types and stage can present markedly different outcomes and responses to therapy, as well as different overall outcomes [26]. One of the biggest criticisms to this system has been its lack of association with biological determinants of prognosis. The advent of high throughput methodologies allowed the reshape of this classification system.

THE ERA OF “MOLECULAR PORTRAITS”

In the past decade, high throughput, microarray based gene expression methods have been applied in breast cancer, in an attempt to further clarify its heterogeneity. The first “molecular portraits” of breast tumours [27] comprised four molecular subtypes, named luminal, HER2-overexpressing, basal-like and normal breast-like (discussed below), based on a hierarchical clustering analysis that used an “intrinsic gene set”. This signature was then validated at the prognostic level [28], which was considered a major breakthrough. These molecular signatures also permit the determination of metastatic propensities [29-31], the molecular basis of histological grade [32] and the identification of prognostic [29-32] and predictive signatures [33].

Although still quite controversial, molecular signatures allowed the subdivision of breast carcinomas into two broad groups, ER-positive and ER-negative, being further molecular classes identified within these two groups. The ER-positive tumours express ER, ER-responsive genes, and other genes that encode characteristic proteins of luminal epithelial cells, being thus denoted as “luminal group”. There seems to be at least two sets of ER-positive breast cancers, commonly defined as luminal A and luminal B, mainly depending on the expression of genes related to proliferation and/or HER2 [34-36]. The second group (ER-negative) was subdivided into three groups: HER2-positive, basal-like tumours and so-called normal breast-like tumours. Recently this ER-negative group was further refined by the inclusion of a new subgroup named claudin-low, which is characterized by loss of genes involved in cell-cell adhesion [37]. HER2-positive tumours express high levels of genes located in the HER2 amplicon on 17q21, including HER2 and Growth Factor Receptor-Bound Protein 7 (GRB7) [38]. Basal-like breast tumours lack ER, PR and HER2 and express genes characteristic of basal/myoepithelial cells. The normal breast-like group was characterized by its resemblance to normal breast tissue samples, however recent studies failed to validate this subtype [39, 40], and attributed the previous findings to technical artefacts, like sample contamination with normal cells.

Importantly these breast cancer subtypes are associated with markedly different clinical outcomes ranging from best prognosis associated with luminal A tumours, to worst prognosis associated with HER2 and basal-like tumours [27, 34, 41]. Furthermore this classification also has predictive value as luminal tumours are expected to be sensitive to endocrine therapy and HER2-overexpressing tumours can be targeted with either monoclonal antibodies or with tyrosine kinase inhibitors. Since basal-like tumours are often triple negative, the majority of these tumours cannot be managed effectively with the current treatments. They are however more sensitive to neoadjuvant chemotherapy (as do HER2-overexpressing tumours) than luminal tumours [42]. These intrinsic subtypes of breast cancers also associate with different sites of distant metastasis. Luminal tumours more frequently metastasize to bone, liver and pleura, whereas basal-like and HER2-overexpressing tumours more frequently metastasize to the brain and lungs [43, 44]. The GEPs have given us prognostic information beyond standard clinical assessment. Several molecular assays have been approved for clinical use, and many more are being developed and validated. For example, the 70 gene MammaPrint®, that has already received approval from the Food and Drug Administration (FDA), has shown prognostic significance mainly in ER-positive breast cancers [31, 45]. Its use is suggested in prognostic prediction in patients below 61 years of age with a stage I or II, node-negative disease with a tumour size below or

equal to 5cm [41]. Another test, the 21-gene OncotypeDx® assay [46, 47], measures the expression of ER and HER2, as well as of ER-regulated transcripts and several proliferation-related genes, with the use of quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR). These measurements are then combined into a quantitative “recurrence score” which can estimate the probability of recurrence at 10 years or can group patients into low, intermediate or high risk categories. Its use is indicated for risk prediction in patient’s ER-positive, lymph node negative treated with tamoxifen and for the identification of patients with a low risk of recurrence that may not need adjuvant chemotherapy [41]. More recently a 50-gene subtype predictor, named PAM50, was developed using both microarray and Q-RT-PCR [40]. It classifies breast cancers into intrinsic subtypes, providing a continuous risk of recurrence score based on the similarity of an individual sample to prototypic subtypes. The advantage of this system compared to the others is that all the subtypes are present, thus not simply reflecting another method of classification that solely reflects ER status.

Nevertheless, detailed histologic, immunohistochemical and additional gene expression analyses are still needed, until a consensus is reached for the true prognostic and predictive value of the “molecular portraits” of breast tumours.

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AIMS AND THESIS OUTLINE

Clinically, breast cancer has for long been recognized as a heterogeneous disease. The high incidence and still high mortality rates, urges the need of accurately predict the prognosis and appropriately select the right therapy for each breast cancer patient. The introduction of high throughput technologies for better stratification of patients enabled the recognition of groups that have already proven their prognostic value. Although the consideration of this classification for breast taxonomy is still far from the initially expected “gold standard” status, it further validated the notion that breast cancer has to be seen as a number of distinct biological entities, prompting the search for other markers that might be used in the future for diagnosis and possibly targeted treatment. In this respect currently breast cancer treatment encompasses three main categories: endocrine therapy, either with tamoxifen or with aromatase inhibitors used in hormone receptor positive tumours (mainly luminal); a humanized monoclonal antibody, trastuzumab, or the tyrosine kinase inhibitor lapatinib, for HER2-positive tumours; and finally chemotherapy, for the treatment of hormone receptor and HER2 negative tumours - basal-like (although chemotherapy can also be used in the other tumour subtypes). Particularly in basal-like tumours there is an increasing need to identify appropriate molecular targets, as the current treatment is considered to be sub-optimal. Thus, a better understanding of the mechanisms that lie in the development and/or progression of basal-like breast carcinomas is of utmost importance.

The main goal of this thesis was to gain insight and try to identify markers that might prove to be more common among basal-like breast carcinomas, as compared with the other subtypes. This will enable a better characterization of this particular subtype and might provide targets for therapy. Furthermore we aimed at determining mechanisms that explain the more aggressive behaviour associated with basal-like breast carcinomas.

For this purpose, **CHAPTER 1** consists of an overview on basal-like breast carcinomas, the development of a tissue bank of invasive breast carcinomas, and the classical immunohistochemical characterization of this breast tumour series, using conventional markers, to allow the classification of breast carcinomas into their respective subtype: luminal A and B, HER2-overexpressing and basal-like.

CHAPTER 2 will present our results regarding the association of the Epidermal Growth Factor Receptor (EGFR) with basal-like breast carcinomas, specifically in the subset of metaplastic carcinomas, also identifying possible mechanisms that lead to this receptor overexpression.

In **CHAPTER 3** we studied two of the most commonly EGFR activated pathways: the phosphoinositide-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways, in

order to correlate downstream targets, and determine if these pathways activation might account, to some extent, to the development/progression of basal-like breast carcinomas.

Finally, in **CHAPTER 4** we sought to refine the knowledge on EGFR signalling networks, to determine systems constituents that might be targeted for therapy or that might be used as predictive markers of response. The identity of Epidermal Growth Factor (EGF)-induced genes that could be potentially involved in mammary cell migration and metastasis, like C-terminal tensin like protein (CTEN) and Synaptojanin 2 (SYNJ2) and also of a negative regulator of EGFR endosomal sorting, the Lateral signalling target protein 2 homolog (hLst2), was addressed.

Each chapter will contain an introduction, materials and methods, results and discussion. If the chapter is based on already published data the different sections will be focused on the most relevant findings. In the end, a summary and conclusions enclosing all chapters, final remarks and future perspectives is included.

CHAPTER 1 – BASAL-LIKE BREAST CARCINOMAS

PAPERS RELATED TO THIS CHAPTER:

I - SILVA F, **CARVALHO S**, MILANEZI F, SCHMITT FC (2008) BASAL-LIKE CARCINOMA OF THE BREAST. ACTA MED PORT 21: 373-8

II - SOUSA B, PAREDES J, MILANEZI F, LOPES N, MARTINS D, DUFLOTH R, VIEIRA D, ALBERGARIA A, VERONESE L, CARNEIRO V, **CARVALHO S**, COSTA JL, ZEFERINO L, SCHMITT F (2010) P-CADHERIN, VIMENTIN AND CK14 FOR IDENTIFICATION OF BASAL-LIKE PHENOTYPE IN BREAST CARCINOMAS: AN IMMUNOHISTOCHEMICAL STUDY. HISTOL HISTOPATHOL 25: 963-974

INTRODUCTION

The impact of GEPs on breast cancer patients' management has probably been one of the most discussed topics on the field in the past ten years. Among the different subtypes encountered [1-4], basal-like breast cancers have drawn particular attention. In the context of breast pathology, the term *basal* has been used to refer to myoepithelial cells, which are basally located, as well to a subgroup of luminal cells that express high molecular weight cytokeratins (CKs) [5]. Basal-like breast carcinomas were so named as the neoplastic cells encountered in this tumour type consistently express genes usually found in normal basal/myoepithelial cells of the breast [2, 6-9]. Characteristically these cells express CK5 (or CK5/6), CK14 and CK17, in addition to other markers including smooth muscle (SM)-specific markers (like SM actin and SM myosin heavy chain), calponin, caldesmon, p63, β_4 integrin, laminin, maspin, CD10, P-cadherin, caveolin 1, nerve growth factor receptor (NGFR), 14-3-3 σ and S-100. They are also typically negative for ER, PR, luminal cytokeratins, desmin and epithelial membrane antigen (EMA) [10, 11]. Although the term *basal-like* reached prime time with the GEPs it had already been recognized for several decades, a subset of breast carcinomas that displayed myoepithelial differentiation and poor prognosis, though at that time no specific designation was used to describe it [12-15].

MORPHOLOGICAL AND CLINICAL FEATURES OF BASAL-LIKE BREAST CARCINOMAS

Basal-like breast carcinomas comprise approximately 8 to 20% of all breast cancers [2, 3, 16-32]. Histologically, the majority of these tumours are ductal NOS, but also, adenoid cystic, medullary and metaplastic carcinomas frequently display basal-like characteristics [33-35]. Some common features of these tumours are younger patient age, high histological grade, marked cellular pleomorphism, high nuclear-cytoplasm ratio, lack of tubule formation, high mitotic index, frequent apoptotic cells, scarce stromal content, pushing borders of invasion and central geographic or comedo-type necrosis. They are also characterized by the presence of metaplastic elements such as spindle cells and squamous metaplasia, presence of a central scar, glomeruloid microvascular proliferation, and a stromal lymphocytic response. It has been suggested that basal-like carcinomas might achieve extraordinarily rapid clinical growth rates [36]. This might account for the frequent association between basal-like carcinomas and the appearance of "interval-cancers" (*i.e.* cancers arising between annual mammograms) [37]. The association between basal-like carcinomas and poor outcome has been remarkably reproducible across many different patient populations [1, 3, 16, 19, 26, 31, 38, 39], being also associated with an aggressive clinical history, development of locoregional and distant metastasis, particularly in the

first 5 years [32], shorter survival and high mortality rate [1, 19, 25, 38-40]. Previous studies have demonstrated that the basal-like phenotype is an independent marker of poor prognosis in breast cancers as a whole [16, 41, 42], in lymph node (LN)-negative [25, 26] and LN-positive groups [43]. Basal-like phenotype was found to predict a particularly aggressive course for patients with grade 3 tumours, LN-negative [31] and in patients with metastatic disease [32]. Noteworthy basal-like tumours present a specific pattern of distant metastasis with an increased propensity for visceral metastasis to brain and lungs [32, 44] and less likelihood to metastasize to bone and liver [19, 22, 32, 45, 46], which is suggestive of a distinct mechanism of metastatic spread.

MOLECULAR FEATURES OF BASAL-LIKE CARCINOMAS

GEPs have revealed expression alterations in particular genes in the different subtypes of breast cancer. The gene expression cluster characteristic of basal/myoepithelial cells included CK5, CK17, β 4 integrin and laminin [2]. Also, the increased copy number variation observed in basal-like breast tumours indicates a higher genetic complexity as compared with the other subtypes, which suggests a greater degree of genetic instability in this subset [47-49]. Basal-like breast carcinomas are relatively enriched for low-level copy-number gains involving several regions, whereas high-level amplifications at any locus are infrequent [47, 50]. Defects on DNA double-strand repair system were proposed for this pattern of genomic changes, which was confirmed by the association of basal-like tumours with a dysfunctional BRCA1 pathway [51-54]. Also, both hereditary *BRCA1*-associated tumours and sporadic basal-like carcinomas have frequent loss of X-inactivation markers [55-57]. As *BRCA1* is rarely mutated in sporadic breast cancers [58] its downregulation in these tumours can be explained by epigenetic events like, promoter methylation [59, 60] or by a dysfunction in the upstream pathways that regulate BRCA1 expression like overexpression of ID4 [51]. Furthermore, the locus at 5q11, which has many checkpoint DNA-repair and tumour suppressor genes, like *MSH3*, *RAD17*, *APC*, *RAD50* and *XRCC4*, was lost on basal-like cancers, but never in the other subtypes [61]. In addition, basal-like breast carcinomas present mutations in *TP53* in up to 85% of the cases [62].

IMMUNOHISTOCHEMICAL CHARACTERIZATION OF BASAL-LIKE CARCINOMAS

To date there is still no internationally accepted immunohistochemical panel to define basal-like carcinomas as there is no specific histological feature that allows its reliable identification in routine practice. As a result, a wide variety of definitions based on the expression of numerous immunohistochemical markers have been used. These definitions include the requirement for triple negativity (*i.e.* ER, PR and HER2 negativity) alongside with a myriad of other markers,

which include CK5 (CK5/6), CK14, CK17, P-cadherin, p63, SMA, Vimentin, c-Kit and EGFR [17, 18, 20, 23, 28, 30, 34, 35, 40, 43, 63-68]. Furthermore there are groups that used basal CKs as solely markers to define basal-like breast carcinomas without restriction to the triple negative tumours [26, 27, 31, 69]. However the most frequently quoted immunohistochemical definition is based on the criteria by Nielsen *et al* [43]. In this study, it was observed that the majority of basal-like breast carcinomas previously defined by GEP, expressed CK5/6, EGFR and c-KIT. Importantly, to date none of the immunohistochemical definitions has been shown to accurately identify all basal-like cancers; however, most of these surrogate markers identify groups of tumours whose characteristics are remarkably similar to those identified by GEP analysis [70].

As basal-like phenotype strongly correlates with the lack of ER, PR and HER2 expression (i.e. triple negative phenotype) [71, 72], extensive research is being conducted in this subset as there is a lack of targeted therapies against it. Still, it is important to refer that triple negative and basal-like are not synonyms [73].

BASAL-LIKE CARCINOMAS: *TO BE OR NOT TO BE* NAMED TRIPLE NEGATIVE

The persistent misconception that triple negative and basal-like tumours are synonyms and that these terms can be used interchangeably is not accurate [73-75]. Triple negative is a term based on clinical assays for ER, PR and HER2, while “basal-like” is considered a molecular phenotype [75]. The original observation that the basal-like phenotype was associated with ER, PR and HER2 negativity, led to the conclusion that in clinical samples, this panel could be used to identify this subtype of tumours [76]. However it is now accepted that not all basal-like tumours have a triple negative phenotype and that not all triple negative tumours have a basal-like GEP [43, 73, 76]. Immunohistochemical analysis of ER, PR and HER2, in basal-like carcinomas, classified according to the intrinsic gene list, has identified that at least 15% of these tumours are positive for one of these three markers [16, 43, 72]. Conversely, not all triple negative tumours cancers appear to be part of the basal-like cluster as defined by microarray analysis [43, 73, 76]. Further analysis of the GEPs suggests that in addition to the basal-like cluster, the triple negative tumours encompass the normal-like subtype as well the newly reported claudin-low tumours [1-4, 16, 71, 77]. In conclusion, basal-like tumours comprise a subset of triple negative tumours, with significant overlap. Still, “triple negativity” should not be used as a surrogate marker for basal-like carcinomas.

In the present chapter results regarding the construction of a tissue bank of invasive breast carcinomas and its full characterization using a panel of biomarkers are presented. This enabled

its classification into the different subtypes: luminal A, luminal B, HER2-overexpressing and basal-like. This series served as basis for the majority of the work presented in the subsequent chapters.

Furthermore the panel used for the identification of basal-like breast carcinomas was refined by addressing the sensitivity and specificity of the main basal markers that have been described, namely CK5, EGFR, P-cadherin, CK14, Vimentin and p63.

MATERIALS AND METHODS

TUMOUR SAMPLES AND TUMOUR BANK CONSTRUCTION

A tumour bank of invasive breast carcinomas was built, using formalin-fixed paraffin embedded tissue samples selected from the archives of Hospital de São João, Porto, Portugal, from January 2001 to February 2006. This resulted in a cohort of 362 invasive breast carcinomas. From all the cases, data was collected regarding the number of the histological exam, date of entry of the patient in the hospital, age of the patient at the time of diagnosis, tumour size, histological grade and type, lymph node status, presence of locoregional and distant metastasis as well as immunohistochemical results for ER, PR and HER2, as all had been performed for diagnostic purposes. All this data was collected through access to the patients' medical records and was then included in an Excel (Microsoft) spread datasheet, preserving the patients' anonymity.

TISSUE MICROARRAY CONSTRUCTION

Of each case at least one paraffin block and its correspondent haematoxylin and eosin (H&E)-stained section slide was collected from the archives of the Department of Anatomic Pathology of Hospital de São João. Representative areas of the different lesions were selected on the H&E-stained sections, and were then marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each selected specimen and deposited into a recipient paraffin block using a tissue microarray (TMA) workstation (TMA builder, Lab Vision Corporation, UK). TMA blocks were constructed, each containing 24 tissue cores, arranged in a 4×6 sector. In each block, at least 3 nonneoplastic breast tissue cores were included as controls as well as 1 core of a histologically characteristic tissue, different from the breast, for slide orientation. After construction, 2 to 3-µm tissue sections were cut and adhered to Superfrost Plus glass slides. An H&E-stained section from each TMA block was reviewed to confirm the presence of morphological representative areas of the original lesions.

IMMUNOHISTOCHEMISTRY AND SUBTYPE CLASSIFICATION

As information regarding ER, PR and HER2 status was available from the anatomic pathology reports, immunohistochemistry was performed for CK5, EGFR and KI67 being the antibodies and the detailed immunohistochemical protocol for CK5 and EGFR described in Paper II and for KI67 described elsewhere [23]. The classification into the different subtypes was based on different studies [16, 23, 78, 79]. Briefly, tumours were classified as: luminal A - if they presented hormone receptor (ER and PR) positivity but lack HER2 overexpression; or were considered to be luminal B - if they expressed ER, PR and overexpressed HER2. Cases lacking ER/PR with overexpression of HER2 were classified as HER2-overexpressing tumors. ER, PR and HER2 negative cases with immunoreactivity for CK5 and/or EGFR, according to Nielsen's criterion [43] were classified as basal-like.

IMMUNOHISTOCHEMICAL CLASSIFICATION

CK5 was considered positive whenever 10% of the neoplastic cells presented cytoplasmic expression. EGFR was scored according to the DakoCytomation HercepTest® as described elsewhere [80]. KI67 was stained on tissue samples and a cut-off value of 10% immunoreactivity in neoplastic cells was used to distinguish between low ($\leq 10\%$) and high ($> 10\%$) nuclear expression. Paper II contains a detailed description of the materials and methods used to address the sensitivity and specificity of the main basal markers.

STATISTICAL ANALYSIS

The Statview software package was used for all statistical analysis. Correlations between categorical variables were performed using the chi-square test and between continuous and categorical values with analysis of variance. Overall survival (OS) was expressed as the number of months from diagnosis to the occurrence of an event (disease-related death). OS analysis was estimated using Kaplan–Meier method and compared using the log-rank test. In all analysis, a significant level of 5% was considered.

RESULTS

BUILDING AN INVASIVE BREAST CARCINOMA TUMOUR BANK ACCORDING TO THEIR SUBTYPE

The construction of the TMAs yielded 21 blocks with the 362 cases of invasive breast carcinomas represented. In each of the TMA sets, immunohistochemistry for CK5 and EGFR was performed, and the cases were classified according to their specific subtype: luminal A, luminal B, HER2-overexpressing and basal-like in relation to the expression of ER, PR, HER2 and the panel of basal markers used (Figure 1.1). Two hundred and fifty six cases were luminal A (70.7%), 38 were luminal B (10.5 %), 35 were HER2-overexpressing (9.7%) and 33 cases were basal-like (9.2%).

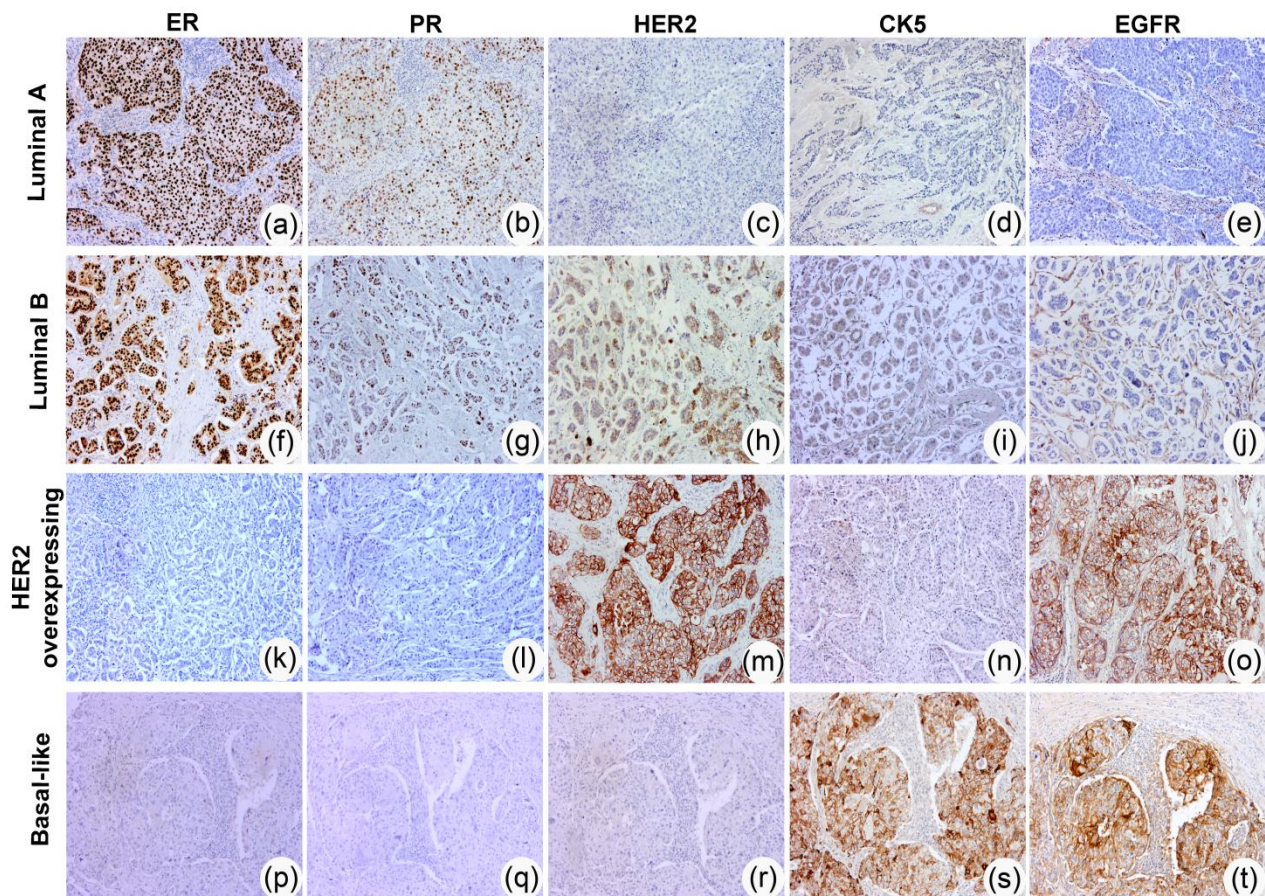


Figure 1.1 – Exemplification of an expression panel of invasive breast carcinomas with the markers used for the distinction into the different subtypes: (a-e) luminal A: ER+/PR+/HER2-, CK5 and EGFR+/-; (f-j) luminal B: ER+/PR+/HER2+, CK5 and EGFR+/-; (k-o) HER2-overexpressing: ER-/PR-/HER2+, CK5 and EGFR+/-; (p-t) basal-like: ER-/PR-/HER2-, CK5 and EGFR+.

The correlations between the different subtypes and the clinicopathological features are summarized in Table 1.1.

Table 1.1 – Association between the different subtypes of breast carcinomas and clinicopathological parameters

Characteristic		Subtypes				<i>p</i>
		Luminal A (n=256)	Luminal B (n=38)	HER2-overexpressing (n=35)	Basal-like (n=33)	
Mean Age (SD)		57.5 (13.4)	55.7 (13.8)	53.0 (11.8)	57.5 (12.4)	NSS
Size (cm)	≤2	132 (52.0%)	15 (39.5%)	13 (37.1%)	16 (50.0%)	NSS
	>2	122 (48.0%)	23 (60.5%)	22 (62.9%)	16 (50.0%)	
Grade	I	74 (29.1%)	5 (13.2%)	1 (2.9%)	1 (3.2%)	<0.0001*
	II	126 (49.6%)	12 (31.6%)	4 (11.4%)	5 (16.1%)	
	III	56 (21.3%)	21 (55.2%)	30 (85.7%)	25 (80.7%)	
Histological type	IDC	180 (70.6%)	29 (76.3%)	29 (85.3%)	28 (87.5%)	0.03*
	ILC	20 (7.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
	IC, mixed type	32 (12.6%)	6 (15.8%)	0 (0.0%)	1 (3.1%)	
	IC, special type	23 (9.0%)	3 (7.9%)	5 (14.7%)	3 (9.4%)	
LN involv.	None	88 (45.6%)	8 (26.7%)	11 (35.5%)	13 (46.4%)	0.048*
	Between 1 and 3	58 (30.1%)	7 (23.3%)	9 (29.0%)	11 (39.3%)	
	More than 3	47 (24.3%)	15 (50.0%)	11 (35.5%)	4 (14.3%)	
Metastasis	None	223 (87.5%)	24 (63.2%)	19 (54.3%)	20 (62.5%)	<0.0001*
	Locoregional	3 (1.2%)	2 (5.3%)	3 (8.6%)	5 (15.6%)	
	Distant	20 (7.8%)	11 (28.9%)	12 (34.3%)	5 (15.6%)	
	Both (locoregional and distant)	9 (3.5%)	1 (2.6%)	1 (2.8%)	2 (6.3%)	
Ki67	Low proliferation	172 (76.1%)	9 (34.6%)	12 (44.4%)	9 (31.0%)	<0.0001*
	High proliferation	54 (23.9%)	17 (65.4%)	15 (55.6%)	20 (69.0%)	

SD – standard deviation; IDC – invasive ductal carcinoma; ILC – invasive lobular carcinoma; IC – invasive carcinoma; LN involv – lymph node involvement; * statistically significant; NSS – not statistically significant.

An association between tumour grade and the different subtypes ($p<0.0001$) was observed. Basal-like and HER2-overexpressing tumours were of higher grade than luminal A and B tumours. Also an association between lymph node involvement ($p=0.048$) and metastasis ($p<0.0001$) was obtained. HER2-overexpressing group presented a higher number of lymph node involvement as opposed to basal-like tumours that presented the lowest number among the subtypes. Regarding the presence of metastasis, basal-like carcinomas were more prone to present locoregional and both locoregional and distant metastasis whereas HER2-overexpressing and luminal B tumours presented a higher number of distant metastasis. Ki67, a nuclear marker

of cell proliferation, was also determined. A strong statistically significance between KI67 and the different subtypes of invasive breast carcinomas ($p < 0.0001$) was also observed. Basal-like tumours presented higher proliferation rates (69% of the cases), as opposed to luminal A tumours that had lower proliferation rates (76.1% of the cases). Neither age of disease diagnosis, nor tumour size, were associated with the different subtypes. There was however a trend for HER2-overexpressing and luminal B tumours, to present a bigger size as compared to luminal A or basal-like tumours.

To evaluate if the different subtypes had an impact on OS a Kaplan-Meier survival curve (Figure 1.2) was performed.

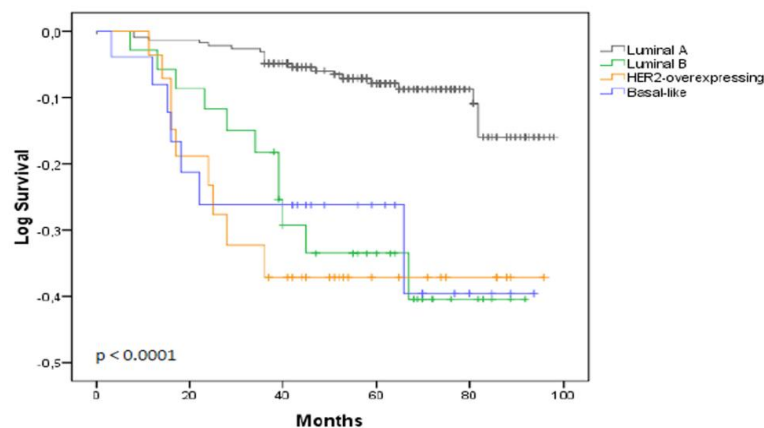


Figure 1.2 – Association of the different subtypes and overall survival, where it can be observed a correlation of luminal A tumours with better outcome whereas luminal B, HER2-overexpressing and basal-like tumours present a worse overall survival ($p < 0.0001$).

There was a clear separation between luminal A tumours and the other subtypes. Luminal A tumours were associated with better outcome. When comparing the three other subtypes, there was no overwhelming difference as all presented similar profiles. Noteworthy all three associated with worse OS.

ADDRESSING THE SENSITIVITY AND SPECIFICITY OF THE MAIN BASAL MARKERS

Paper II contains a detailed description of the results. Hence only a brief description of the key findings will be mentioned.

When determining the specificity and sensitivity of the main basal markers CK5, EGFR, P-cadherin, CK14, Vimentin and p63 it was possible to observe that P-cadherin and CK5 presented a higher sensitivity (91.6% and 67.8% respectively) while EGFR, CK14 and Vimentin could be

considered more specific markers (97.1%, 98.6% and 86.9% respectively). p63 however, was not related with basal-like phenotype. The combination of CK5 with P-cadherin, CK14 or Vimentin was a reliable option for determining basal-like tumours when compared to the combination of CK5 and EGFR. In the subset of triple negative tumours also negative for CK5 and EGFR (named “unclassified” according to Nielsen’s criterion), P-cadherin was expressed in 40.3% of cases, rescuing a subset of putative basal-like carcinomas from the otherwise unclassified tumours.

DISCUSSION

The identification and validation of markers and biological pathways that allow an accurate determination of prognosis, response or even resistance to therapy is one of the most promising directions of cancer research. The first GEPs described by Perou *et al* [2] altered our view of breast cancer, providing a new tool for molecular diagnosis. However, GEPs still present some drawbacks as to their readily application in the clinical setting, as it is still an expensive technique and not widespread in central hospitals. To overcome these limitations several groups attempted to define surrogate immunohistochemical markers that enabled the identification of the subtypes defined by GEPs, in particular basal-like tumours, as this subset still does not have a target-based therapy and presents poor prognosis. In this first chapter, the construction of an invasive breast carcinoma tissue bank is described. The aim was to create a tool for the assessment of molecular markers. Three hundred and sixty two cases of invasive breast carcinomas were retrieved from the archives of the Department of Anatomic Pathology of the Hospital de São João and were included in 21 TMAs. The invasive breast carcinomas were divided into the subtypes described in the GEPs: luminal A, luminal B, HER2-overexpressing and basal-like. Information on ER, PR and HER2 (already determined for diagnostic purposes) as well as CK5 and EGFR (as proposed by the Nielsen’s criterion), was used. Based on this classification 256 luminal A cases (70.7%), 38 luminal B cases (10.5 %), 35 HER2-overexpressing (9.7%) and 33 basal-like (9.2%) were obtained. When correlating the different subtypes with clinicopathological parameters it was clear that basal-like and HER2-overexpressing tumours were of higher grade, an observation in agreement with other publications [11, 81]. An association between the number of lymph nodes involved and presence of metastasis was also observed. HER2-overexpressing tumours presented a higher number of cases with lymph nodes involved as opposed to basal-like tumours. This lack of association between basal-like tumours and lymph node involvement is still unsettled, since the worse prognosis of basal-like tumours was described in both lymph node negative [25,

26] and lymph node positive [43] settings. Regarding metastasis, basal-like breast carcinomas were more prone to the development of locoregional and both locoregional and distant metastasis. In accordance with other published data [32], HER2-overexpressing and luminal B tumours presented a higher number of cases with distant metastasis. Cell proliferation, as determined by KI67 staining was also assessed, since breast carcinomas expressing high levels of KI67 are associated with worse outcomes [82-84]. In accordance to the literature, basal-like carcinomas presented higher proliferation rates [11], a fact that can explain the already mentioned overrepresentation of this subtype among interval-breast cancers [37]. There was no association with age of onset of the disease as no significant difference between the subtypes was observed. This result was unanticipated, as basal-like carcinomas are more frequently associated with younger ages of onset [11]. There were also no differences regarding tumour size, a trait also common in other studies [19, 32]. Regarding OS, the observation that luminal A tumours present better prognosis as opposed to luminal B, HER2-overexpressing and basal-like that present worse prognosis, is in agreement with published data [1, 3, 43, 77].

The usefulness of P-cadherin, CK14 and Vimentin as biomarkers to distinguish basal-like breast carcinomas (Paper II) was also demonstrated. When associated with CK5 these three markers were able to reliably identify the basal phenotype. P-cadherin had already been described in invasive and *in situ* breast carcinomas with worse prognosis. There is also an established correlation between its expression and EGFR, HER2 as well as an inverse correlation with ER, strengthening its value as a poor prognostic marker [78, 85-88]. Furthermore, P-cadherin was able to rescue possible basal-like breast carcinomas among triple negative tumours that also do not express either CK5 or EGFR. Both CK14 and Vimentin have also been proposed as basal markers. The former is associated with worse prognosis and with morphological features of basal-like tumours [17, 27, 31, 45] and the later was suggested to integrate the panel of antibodies used for the identification of basal-like breast carcinomas due to its differential expression determined by GEPs in basal-like breast carcinomas [65].

Currently the correspondence between GEPs and immunohistochemical methods for the identification of basal-like breast carcinomas is less than perfect. However, this rediscovery of breast cancer classification gave rise to new inputs that enabled a better understanding of the biological drivers of this complex disease. Although logistic complexities limit the feasibility of combining five or more immunohistochemical markers in routine clinical practice, this “digging” for a specific marker for basal-like breast carcinomas identification allowed the recognition of several

potential targets that might be used for the treatment of at least a small subset of basal-like breast carcinomas. One of these targets is the EGFR.

KEY FINDINGS

- Establishment of an invasive breast carcinoma tumour bank classified according to the different subtypes of breast carcinomas: luminal A, luminal B, HER2 overexpressing and basal-like;
- Basal-like breast carcinomas were grade III, had higher proliferation rates and frequently presented both locoregional and distant metastasis;
- P-cadherin, CK14 and Vimentin proved to be useful biomarkers in the identification of basal-like breast carcinomas;
- P-cadherin was able to rescue possible basal-like breast carcinomas among triple-negative cases that do not express either CK5 or EGFR.

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CHAPTER 2 – THE IMPORTANCE OF EGFR IN BASAL-LIKE BREAST CARCINOMAS

PAPERS RELATED TO THIS CHAPTER:

III - REIS-FILHO JS*, MILANEZI F*, **CARVALHO S***, SIMPSON PT, STEELE D, SAVAGE K, LAMBROS MB, PEREIRA EM, NESLAND JM, LAKHANI SR, SCHMITT FC (2005) METAPLASTIC BREAST CARCINOMAS EXHIBIT EGFR, BUT NOT HER2, GENE AMPLIFICATION AND OVEREXPRESSION: IMMUNOHISTOCHEMICAL AND CHROMOGENIC IN SITU HYBRIDIZATION ANALYSIS. BREAST CANCER RES 7: R1028-35

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INTRODUCTION

BIOLOGY OF THE HER FAMILY OF RECEPTORS

Over the past decades, receptor tyrosine kinases (RTKs) appeared as prominent targets for cancer therapy. Among this broad class of receptors, the HER family (also named ErbB), can be considered the prototypic founder sub-group of the RTK super-family, which includes 18 other small sub-groups of related receptors [1]. The HER family of receptors are important mediators of cell transformation, proliferation, maintenance and survival, with expression in various tissues of epithelial, mesenchymal and neuronal origins [2, 3]. Signalling through these receptors plays a critical developmental role in cell fate determination in many organ systems [3]. This family comprises four type I growth factor receptors that share a high degree of homology (HER1 to 4 also known as ErbB1 to 4, being HER1 usually designated EGFR) [4]. The receptors are composed of an extracellular ligand-binding domain, followed by a transmembrane domain, a small intracellular juxtamembrane domain preceding the kinase domain, and a C-terminal tail, on which the docking sites for phosphotyrosine-binding effectors molecules are found [4]. Two out of four HER proteins, namely EGFR and HER4 are autonomous; when bound by a ligand growth factor they undergo dimerization and generate intracellular signals that culminate in cell proliferation, migration or differentiation [1]. The two other receptors, HER2 and HER3, are non-autonomous [1]; HER2 is a ligandless receptor [5], whereas HER3 presents an impaired kinase function [6], thus it is unable to generate signals by itself. Interestingly, although disabled, HER2/HER3 heterodimers are capable of forming the most potent signalling module of the HER receptor family [7]. Their ligands can be divided into three groups: (1) EGF, transforming growth factor α (TGF α) and amphiregulin that bind exclusively to EGFR; (2) betacellulin, heparin-binding EGF-like growth factor and epiregulin, which show dual-specificity, binding both EGFR and HER4; and neuregulins (NRGs), which are divided into two groups, mostly based on their ability to bind HER3 and HER4 (NRG1 and 2) or only HER4 (NRG3 and 4) [3, 7, 8]. In their inactive state HER receptors are in a monomeric state. Upon ligand binding homo or heterodimerization can occur, leading to the activation of the kinase domain. The phosphorylated residues will then serve as docking sites to several proteins that will lead to the activation of downstream signalling pathways [2, 9]. The major downstream signalling pathways of the HER family are the RAS-RAF-MAPK and the PI3K. Activation of RAS and/or PI3K initiates a multistep phosphorylation cascade that leads to the activation of MAPK or of the protein serine/threonine kinase AKT [3, 7].

Dysregulation of the HER signalling network is implicated in multiple human pathologies, including cancer, of which EGFR and HER2 are considered to be major players.

MECHANISMS OF RECEPTOR ACTIVATION

HER RTK activation is related to several hallmarks of cancer cells, including excessive and autonomous growth, invasiveness, apoptosis evasion, insensitivity to inhibitory growth signals and stimulation of angiogenesis, as elegantly described by Hanahan and Weinberg [10].

Increased activation of signalling pathways as a result of HER activation might be achieved through receptor overexpression, which can occur through: (1) mechanisms of gene amplification; (2) as a result of activating mutations; (3) decreased levels of phosphatases; (4) increased coexpression of receptor ligands (5) heterodimerization and cross-talk between the different members of the HER family; as well as (6) through interaction with other heterologous systems [11, 12]. EGFR protein overexpression has been observed in a variety of human malignancies, including head and neck, lung, colon and breast cancer as well as in gliomas [12]. Mutant forms of EGFR have been found in several human tumours, and interestingly these alterations cluster in specific areas of the gene, leading to the assumption that “mutational hotspots” entail elements of special function and regulatory importance [13] that might dictate the aetiology of certain tumours. The most common mutated form of EGFR is, EGFRvIII, an in frame deletion of the extracellular domain (ECD) that bypasses the need of a ligand, leads to a constitutive dimerization of the receptor and is highly tumorigenic [14]. It is frequently observed in brain tumours although it can also be observed in other malignancies like, lung, breast and ovarian cancers [15-18]. Mutations in the kinase domain are frequently encountered in non small cell lung carcinomas (NSCLC). The majority are point mutations that render a constitutive activation of this domain even in the absence of upstream events like ligand-binding or dimerization [19, 20]. Another mechanism that might lead to receptor overexpression, which is commonly forgotten, is the regulation of the endocytic removal of active ligand–receptor complexes from the cell surface, and their sorting to degradation or recycling [21]. Protein ubiquitylation seems to play an important role in this setting [21, 22].

HER2 overexpression is frequently observed in human carcinomas, but the association between overexpression and clinical parameters is best defined in breast and gastric cancers [23-25]. Gene amplification is by far the most prevalent mechanism associated with protein overexpression in malignant tumours, particularly in breast carcinomas. A low percentage of mutations in the tyrosine kinase domain of *HER2* (2.2 – 4.3%) have been described in lung

adenocarcinomas [26, 27]. HER2 is subjected to an additional type of regulation, mediated by molecular chaperones, particularly heat shock protein (HSP) 90. The chaperone system controls the biogenesis, stability and activity of several proteins [28-30]. HER2 is strongly coupled to HSP90 as opposed to wild type EGFR that only weakly interacts with HSP90. The role of HSP90 in the regulation of HER2 has been attributed to stabilization of the receptor at the cell surface, such that upon inhibition of HSP90 with ansamycins the receptor undergoes proteasomal degradation [28]. Interestingly, mutant forms of EGFR require HSP90 for conformational maturation, contrary to the observed for wild type EGFR, as proven by their sensitivity to degradation upon HSP90 inhibition [31-33].

HER THERAPEUTICAL TARGETING

Targeting the HER family of receptors might be achieved by two main therapeutical strategies: inhibition of the ECD with monoclonal antibodies (mAbs) or through inhibition of the tyrosine catalytic domain with small-molecule tyrosine kinase inhibitor (TKI) [34]. The first anti-HER therapy approved by the Food and Drug Administration (FDA) was trastuzumab (Herceptin®) for the treatment of HER2-positive breast cancer. It was initially approved as a first-line treatment in combination with paclitaxel for HER2-positive metastatic breast cancer [35]. The benefit of this approach in patients with metastatic disease and the poor prognosis associated with HER2-positive breast cancer encouraged clinical trials of adjuvant treatment with trastuzumab [36, 37]. Response rates to single-agent trastuzumab range from 12-34% in metastatic breast cancer [38, 39]. Since trastuzumab approval in the adjuvant setting in 2006, significant improvements in disease-free survival rates were also achieved in patients with early-stage HER2-overexpressing breast cancer [40, 41]. EGFR's extracellular domain can be similarly targeted using specific antibodies, that inhibit ligand-binding and accelerate receptor down-regulation following antibody-directed clustering and aggregation [42]. Two antibodies have already received FDA approval: Cetuximab (C225, Erbitux®) and Panitumumab (Vectibix®). Cetuximab is a humanized mouse antibody approved for the treatment of patients with metastatic colorectal carcinomas and patients with advanced or recurrent head and neck cancer. Panitumumab is a fully human EGFR antibody approved for the treatment of metastatic colorectal carcinomas with disease progression despite prior treatment. Recently FDA incorporated the need for K-RAS testing into Erbitux® and Vectibix® labels, as wild type K-RAS is needed for the efficacy of the treatment [43-45]. Another promising approach was the development of small molecules that bind to the ATP-binding pocket of the kinase domain, thereby inhibiting its enzymatic activity. These TKIs mimic adenosine triphosphate (ATP), which serves as phosphate source for the phosphorylation process. These

TKIs can bind either reversibly or irreversibly, and can target EGFR alone, other HER family members or even other tyrosine kinases as well [13]. Erlotinib (OSI774, Tarceva®) and gefitinib (ZD1839, Iressa®) are two reversible EGFR-specific TKIs. Erlotinib has been approved for the treatment of locally advanced or metastatic NSCLC that failed at least one prior chemotherapy regimen, or in combination with gemcitabine for treatment of locally advanced, unresectable, or metastatic pancreatic cancer [46-49]. Although gefitinib was the first EGFR TKI to become commercially available, the modest response rates observed, and its failure in prolonging survival rates, precluded its removal from the market in the United States and European Union [50]. However, as Asian patients seemed to benefit from its usage, Gefitinib is currently being used as a second or third-line option for the treatment of refractory NSCLC in East Asia [51]. A third approved compound, lapatinib (Tykerb®) inhibits both EGFR and HER2. Lapatinib was already indicated in combination with capecitabine (Xeloda®) for the treatment of patients with advanced or metastatic breast cancer whose tumours overexpress HER2 [52], and has recently been approved in combination with letrozole (Femara®) for the treatment of postmenopausal women with hormone receptor positive metastatic breast cancer that overexpresses HER2 [53].

The assessment of HER2 overexpression and/or amplification by immunohistochemistry and *in situ* hybridization techniques is currently part of the routine practice for breast cancer management, being the therapeutical options for HER2 already taken into account when choosing the available treatment schemes. EGFR, on the other hand, only recently has assumed a relevant place in breast cancer after its association with the basal-like phenotype, either in invasive carcinomas [54, 55] or in DCIS [56-58].

EGFR IN BASAL-LIKE BREAST CARCINOMAS

The association of EGFR with basal-like breast carcinomas set basis for the attempt to treat this tumour subset with the currently available anti-EGFR target therapies. As previously stated, basal-like breast carcinomas often have a triple-negative phenotype which results in a lack of efficient management of these patients with the available targeted treatments (trastuzumab and hormonal therapy) [59]. One of the first definitions of basal-like carcinomas that suggested the inclusion of EGFR in the panel of markers was proposed by Nielsen *et al* [54] after the observation that EGFR was overexpressed in 57% of the basal-like tumours, previously determined by GEPs. The use of a panel defined by the lack of ER and HER2 expression and expressing CK5/6 and EGFR proved to have a specificity of 100% and a sensitivity of 76% for the identification of basal-like breast carcinomas [54], being this criteria also adopted by many other groups [60].

In the present chapter EGFR was evaluated as a basal-like marker. For that the invasive breast carcinoma tumour bank samples described in Chapter 1 as well as a series of metaplastic breast carcinomas previously shown to present basal-like features [61-64], were used. In the metaplastic breast carcinomas, together with the expression analysis, both copy number alterations and presence of activating mutations were evaluated, as these might preclude the use of targeted therapies against EGFR.

MATERIALS AND METHODS

Paper III contains a detailed description for the immunohistochemical protocol and scoring system used to evaluate EGFR.

The protocols for copy number assessment, by chromogenic in situ hybridization (CISH), and for the analysis of mutational status are described in Papers III and IV respectively.

RESULTS

EGFR EVALUATION IN THE SERIES OF INVASIVE BREAST CARCINOMAS

EGFR expression was assessed in the invasive breast carcinoma series (362 cases – described in Chapter 1). Although EGFR was used as a marker of basal-like carcinomas, according to Nielsen's criteria [54], we decided to validate its wider expression among basal-like tumours, as compared to the other subtypes. Also, the relation between EGFR expression and a more aggressive biological behaviour, not only basal-like related, was also addressed. EGFR expression was detected in 20 out of 353 cases (5.7%). It was more frequently expressed in basal-like breast carcinomas than in the other subtypes (Table 2.1). A statistically significant association between EGFR expression and tumour grade ($p=0.0002$), proliferation index as assessed by Ki67 ($p<0.0001$), presence of metastasis ($p=0.015$), ER ($p<0.0001$) and HER2 ($p=0.0003$) was found. EGFR positive tumours were more frequently higher grade, more proliferative and were associated with ER negativity.

Table 2.1 – Association between EGFR expression in invasive breast carcinomas and clinicopathological parameters

Characteristic		EGFR		<i>p</i>
		Positive	Negative	
Subtype	Luminal A	2 (10.0%)	251 (75.4%)	<0.0001*
	Luminal B	6 (30.0%)	27 (8.1%)	
	HER2-overexpressing	4 (20.0%)	31 (9.3%)	
	Basal-like	8 (40.0%)	24 (7.2%)	
Size	≤2 cm	8 (40.0%)	165 (49.7%)	NSS
	>2 cm	12 (60.0%)	167 (50.3%)	
Grade	I	1 (5.0%)	79 (23.9%)	0.0002*
	II	3 (15.0%)	139 (42.0%)	
	III	16 (80.0%)	113 (34.1%)	
LN involv.	None	8 (44.4%)	110 (42.6%)	NSS
	Between 1 and 3	2 (11.2%)	82 (31.8%)	
	More than 3	8 (44.4%)	66 (25.6%)	
Metastasis	None	11 (55.0%)	268 (80.5%)	0.015*
	Locoregional	3 (15.0%)	10 (3.0%)	
	Distant	5 (25.0%)	43 (12.9%)	
	Both (locoregional and distant)	1 (5.0%)	12 (3.6%)	
Ki67	Low proliferation	4 (21.1%)	196 (69.0%)	<0.0001*
	High proliferation	15 (78.9%)	88 (31.0%)	
ER	Positive	8 (40.0%)	278 (83.5%)	<0.0001*
	Negative	12 (60.0%)	55 (16.5%)	
HER2	Positive	10 (50.0%)	58 (17.4%)	0.0003*
	Negative	10 (50.0%)	275 (82.6%)	

LN involv – lymph node involvement; *statistically significant; NSS – not statistically significant

Although not statistically significant, there was a tendency for EGFR positive tumours to be larger. EGFR expression showed no association with OS (Figure 2.1). However patients with tumours expressing EGFR had a trend toward shorter OS.

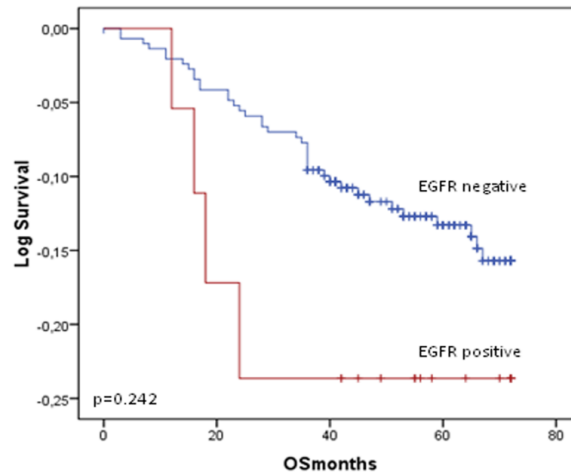


Figure 2.1 – Prognostic impact of EGFR overexpression in invasive breast carcinomas on OS

EGFR EXPRESSION, COPY NUMBER ALTERATIONS AND MUTATIONAL ANALYSIS IN METAPLASTIC BREAST CARCINOMAS

Papers III and IV contain a detailed description of the results on EGFR expression, copy number alterations and mutational analysis in metaplastic breast carcinomas. Hence only a brief description of the key findings will be mentioned.

Metaplastic breast carcinomas account for up to 3.7% of all breast carcinomas. They were consistently associated with a basal/myoepithelial phenotype [61-64] which suggested that they could be part of the basal-like carcinomas spectrum. In Papers III and IV the prevalence of EGFR expression, *EGFR* gene amplification as well as presence of activating mutations in the tyrosine kinase domain of *EGFR* in metaplastic carcinomas of the breast was investigated. In Paper III, EGFR overexpression was observed in 19 out of 25 (76%) of the cases. Of these 19 cases, seven presented *EGFR* gene amplification (Figure 2.2).

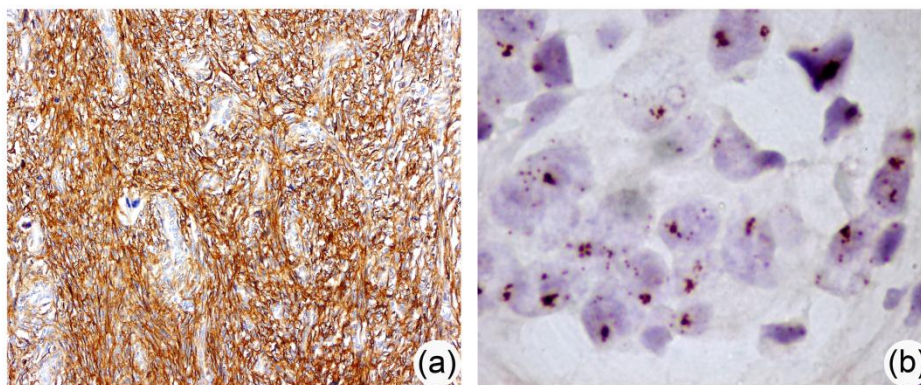


Figure 2.2 – EGFR expression and gene copy number assessment in a metaplastic breast carcinoma spindle cell type: (a) EGFR overexpression 3+ and (b) gene amplification.

There was no association between either EGFR overexpression or amplification and clinicopathological features. Patients with tumours harbouring EGFR amplification had a trend towards shorter DFS and OS.

In Paper IV, the series of metaplastic breast carcinomas was increased and EGFR overexpression was observed in 32 out of 47 (68%) cases. EGFR overexpression was associated with the type of metaplastic elements observed (Table 2.2). Carcinomas with homologous differentiation (either spindle or squamous) were more frequently positive for EGFR than carcinomas with heterologous differentiation.

Table 2.2 – Correlation between type of metaplasia and EGFR overexpression or gene copy numbers in metaplastic breast carcinomas

Type of metaplasia	EGFR IHC		<i>p</i>	EGFR CISH		<i>p</i>
	Negative (0/1+)	Positive (2+/3+)		No amp.	Amp.	
Homologous (n=36)	8	28	0.02	25	11	0.05
Heterologous (n=11)	7	4		11	0	

EGFR amplification was found in 11 out of the 32 (34%) cases. Once again no association between either EGFR overexpression nor amplification and clinicopathological features was observed. Also, none of the 47 metaplastic breast carcinomas presented activating mutations in the tyrosine kinase domain (exons 18, 19, 20 and 21).

DISCUSSION

Activation of HER family of RTKs instigates an intracellular web of events that can drive dramatic cellular responses, such as proliferation and migration [2, 8]. As expected, these receptors are frequently altered in cancer being important factors for tumour initiation and progression. Clinical studies conducted over the past years have established the HER family, particularly EGFR and HER2, as appealing target molecules for cancer therapy. They are frequently expressed in solid tumours, their increased expression is related with poorer clinical outcomes and both are transmembrane receptors, which facilitate their blockage by immunotherapy. In breast cancer HER2 is an essential step in the diagnostic workup. In the updated ASCO recommendations for the use of tumour markers in breast cancer, HER2 has assumed a relevant position, alongside with hormonal receptor status [65]. EGFR on the other hand only recently gained a relevant position in breast cancer after its association with basal-like carcinomas. This subtype of breast carcinomas has attracted great attention as it presents an aggressive clinical behaviour. They are frequently triple negative, which therapeutically means they cannot be managed effectively with the currently available target treatments [59]. As EGFR is already a successful therapeutic target in other tumours like colorectal, pancreatic, and NSCLC [43, 45, 47-49] the rationale that basal-like breast carcinomas could be targeted with anti-EGFR drugs seemed more than reasonable. Based on this, EGFR was assessed in the invasive breast carcinomas series (described in Chapter I), also enabling its validation as a good basal-like marker. EGFR expression was detected in 20 out of 353 cases of invasive breast carcinomas. It was more frequently observed in basal-like breast carcinomas than in the other subtypes, and regardless of the tumour subtype there was a tendency for EGFR positive tumours to be associated with worse OS. Moreover EGFR expressing tumours were associated with higher grade, presented a higher proliferation rate and were associated with ER negativity. There was no difference related to HER2, as EGFR expression was observed in both HER2-positive and HER2-negative tumours. As the number of EGFR positive tumours in luminal B and HER2-overexpressing tumours was low, we were unable to determine if co-expression of both receptors (EGFR and HER2), could be related to worse OS as this premise was already observed by DiGiovanna *et al* [66] where the authors provided the first clinical evidence that EGFR expression was linked to activation of HER2 in human breast cancers. These authors further showed that the adverse prognostic value of HER2 was only observed when the receptor was phosphorylated or coexpressed with EGFR. Although we are aware that the percentage of EGFR positive cases in our invasive breast carcinomas series is quite low, it further emphasizes the need to define consensual methodologies and scoring

systems for EGFR evaluation. This lack of standardization is patent in the descriptions of EGFR expression in breast cancer that range from 14 to 91% [67], mainly due to different methodologies applied for EGFR detection (either radioligand binding assays, immunohistochemistry, autoradiography and measurements of EGFR transcripts), and to different antibodies and scoring systems used for EGFR assessment. For the present study, EGFR classification was based on the Herceptest® scoring system where cases 0 and 1+ are considered to be negative and only the 2+ and 3+ are considered positive. Also the results with metaplastic breast carcinomas (discussed below) suggest that in breast, EGFR is probably more related to specific histological types not presenting a throughout expression in invasive breast carcinomas.

Metaplastic breast carcinomas, were already associated with basal-like phenotype [61-64], and frequently show EGFR overexpression at both the protein and mRNA levels [68, 69]. In metaplastic breast carcinomas, EGFR was overexpressed in 68% of the cases being gene amplification the underlying genetic mechanism in 34% of them. Also, as many cases that presented EGFR overexpression did not have gene amplification the occurrence of activating mutations was determined, as these were also described to be associated with receptor overexpression [70]. Exons 18 to 21 that codify the tyrosine kinase domain and are hot-spot regions for EGFR gain of function mutations (for example in lung carcinomas) [19, 71] were screened. No activating mutations were identified in the tyrosine kinase domain, which was in agreement with previous studies that analysed the presence of activating mutation in breast carcinomas [72]. The mechanism for EGFR overexpression in the majority of metaplastic breast carcinomas still remains elusive. It might be due to the maintenance of myoepithelial phenotype or could even be part of the transcriptomic programming of differentiation of this cell type, given that it is consistently expressed not only in tumours with basal and/or myoepithelial differentiation [54, 73-75] but also in normal myoepithelial cells [76].

The efficacy of anti-EGFR therapies in basal-like breast carcinoma cases should be tested given that: (1) basal-like breast carcinomas currently do not have a target therapy; (2) EGFR plays a role in this subtype (even if in a more histological type specific manner); (3) tumours, such as lung tumours, presenting EGFR amplifications are reported to respond to tyrosine kinase inhibitors [77-79]; and that (4) a subset of metaplastic breast carcinomas present these amplifications. However up to now, no therapy against EGFR has proven its efficacy in breast cancer clinical setting. This is attributed to some extent, to the lack of knowledge in the level of EGFR expression needed for a tumour to obtain response to the inhibitors. As breast cancer cell lines with either low or high EGFR expression levels were inhibited by gefitinib, EGFR expression was

not required to enrol patients in the clinical trials [80-83]. Also, EGFR expression is normally heterogeneous, which conceivably can result in a response to inhibition in the EGFR-positive cells and a lack of it in the EGFR-negative [83]. In the majority of breast cancer clinical trials with EGFR inhibitors, skin was normally used as a surrogate tissue to predict inhibition of EGFR activation in the tumour [83-85]. Skin is easily accessible, with high levels of EGFR expression [85], and pharmacodynamic tests have been developed to examine alterations in EGF/EGFR-inducible responses after drug administration. Furthermore, the expression of EGFR inducible molecules such as phospho MAPK, p27, p-STAT3 and others can also be studied in skin biopsies [84, 85]. In one of the trials, expression of EGFR, p-EGFR and the downstream targets, p-AKT, p-ERK1/ERK2 as well as proliferation index determined by Ki67 and the cell cycle regulator p27 were assessed in both skin and tumour samples [83]. In the skin, gefitinib inhibited p-EGFR, p-ERK1/ERK2 as well as decreased the expression of Ki67, while increasing the expression of p27. However when analysing the corresponding tumours the results were not in agreement. Although in tumours gefitinib also inhibited MAPK phosphorylation, this was independent on EGFR phosphorylation status, as this inhibition occurred in both EGFR-positive and EGFR-negative tumours. Of course, this could be due to technical problems as immunohistochemistry could have failed the detection of low levels of EGFR; however gefitinib also failed to inhibit the activated form of AKT in tumours. Noteworthy Ki67 was not decreased [82, 83] nor p27 levels increased [83]. This lack of meaningful clinical activity could be attributed to a lack of tumour dependency on EGFR [83] even when the receptor is overexpressed. Another important determinant in predicting response to EGFR therapies could be the presence of activating mutations, which, as shown in the present work as well as in other studies [19, 72], are a rare event in breast carcinomas. Another important finding that will be discussed in more detail in Chapter 3, is the lack of PTEN expression in basal-like carcinomas. This might also preclude the use of anti-EGFR therapies in this tumour subtype, as observed for trastuzumab in HER2-positive tumours [86-88]. Further analyses on the specific role of EGFR in breast cancer, particularly in basal-like breast carcinomas with EGFR overexpression, are warranted. Currently there are several undergoing clinical trials with EGFR inhibitors, most of them in metastatic breast cancer. In triple negative breast carcinomas in particular, the tyrosine kinase inhibitor erlotinib will be tested in EGFR-positive, tumours that will be assessed not only for protein expression but also for gene amplification. Patients with metaplastic carcinomas will be included, if the EGFR overexpression criterion is met (ClinicalTrials.gov identifier: NCT00739063). Also EGFR positivity is also an inclusion criterion in another study that will evaluate the efficacy of erlotinib in triple negative tumours (ClinicalTrials.gov identifier: NCT00597597). These trials will help to determine if the

previous disappointing results were mainly due to the use of unselected patient populations (not selected by EGFR status). For the other clinical trials with triple negative breast carcinomas, tissue block availability for EGFR testing is recommended but will not exclude patients from participating in the trials (ClinicalTrials.gov identifiers: NCT00420329, NCT00492375, NCT00894504).

Other important players might masquerade the efficacy of anti-EGFR therapies in breast cancer, for which the identification of relevant oncogenic pathways might be a fundamental prerequisite for their further comprehension. The observation that EGFR as well as many other basal-like gene products activate similar signalling pathways like PI3K and MAPK [89], suggests that these pathways might have a central role in the pathogenesis of these tumours.

KEY FINDINGS

- EGFR expression is more frequently observed in basal-like breast carcinomas than in other subtypes;
- EGFR is overexpressed in 68% of metaplastic breast carcinomas and in 34% of these gene amplification was the underlying mechanism.

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CHAPTER 3 – SIGNALLING PATHWAYS IN BASAL-LIKE BREAST CARCINOMAS

PAPERS RELATED TO THIS CHAPTER:

VI – **CARVALHO S**, MILANEZI F, COSTA JL, AMENDOEIRA I, SCHMITT F (2010) PIKING THE RIGHT ISOFORM: THE EMERGENT ROLE OF THE P110BETA SUBUNIT IN BREAST CANCER. *VIRCHOWS ARCH* 456: 235-43

VII – **CARVALHO S**, SCHMITT F (2010) THE POTENTIAL ROLE OF PI3K INHIBITORS IN THE TREATMENT OF BREAST CANCER. *FUTURE ONCOLOGY* [IN PRESS]

INTRODUCTION

Signal transduction systems are often exploited for malignancy. They comprise growth factors, transmembrane receptor proteins, as well as cytoplasmic secondary proteins that orchestrate cellular responses that range from proliferation, to differentiation, angiogenesis, apoptosis amongst others [1]. The PI3K and the RAS-RAF-MAPK signalling pathways play central roles in the signal transduction networks that govern both tumour initiation and progression (Figure 3.1) [2]. This role is further highlighted by the high frequency of alterations, either genetic or epigenetic, found in pathway effectors, like, *PIK3CA*, *AKT*, *PTEN*, *RAS* and *RAF* [3-11].

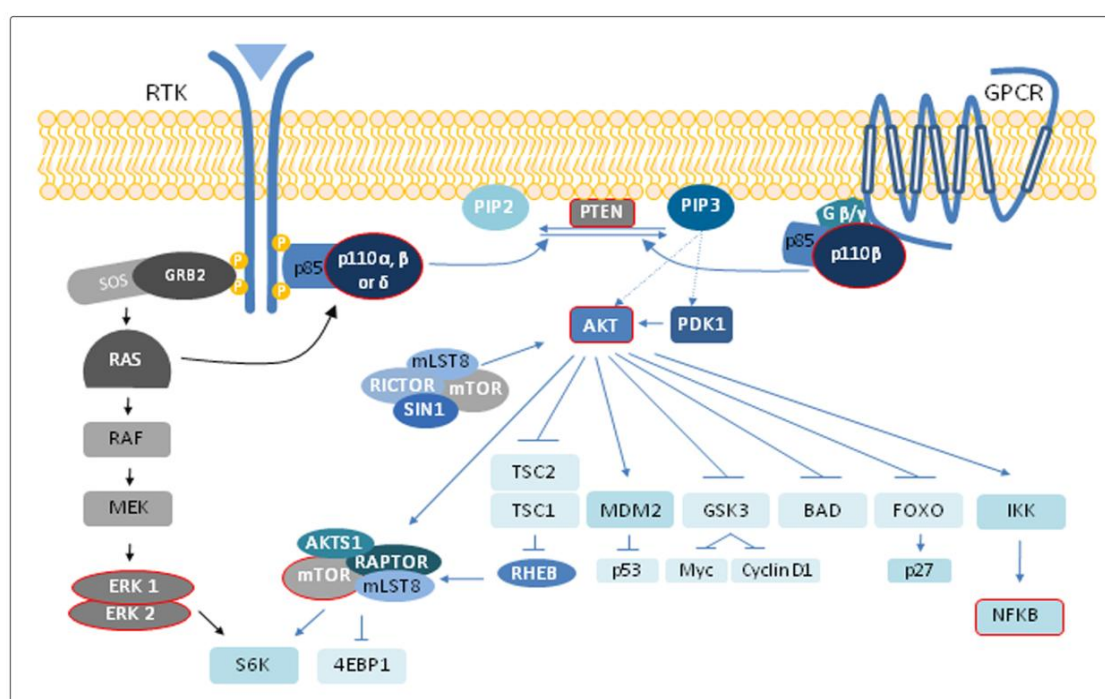


Figure 3.1 – The PI3K and MAPK signalling pathways. PI3K signalling pathway has a role on mechanisms such as cell growth, survival and metabolism. Following growth factor stimulation and subsequent activation of RTKs, class I_A PI3Ks are recruited to the membrane by direct interaction with p85 subunit with the activated receptors or by interaction with adaptor proteins associated with the receptors. p110β-containing enzymes might also be activated by GPCRs. The activated p110 catalytic subunit will then convert PIP₂ to PIP₃ at the membrane, providing docking sites for PDK1 and AKT. PDK1 phosphorylates and activates AKT, which elicits a range of downstream signalling events. PTEN dephosphorylates PIP₃, and therefore terminates PI3K signalling. The phosphorylated tyrosine residues of RTKs can also serve as docking sites for various adaptor proteins such as GRB2. These adaptor proteins recruit further effectors, including RAS-GEFs such as SOS that stimulate the exchange of GDP for GTP on RAS. The RAS effector RAF will then phosphorylate the dual-specificity MEK kinases, which in turn phosphorylate and activate the ERK1 and ERK2. ERK phosphorylates a wide variety of substrates that mediate cell growth and cell-cycle entry. In

addition to transcription factors ERK phosphorylates and activates the S6K and MNK families. PI3K is also an effector of RAS. Highlighted in red are the proteins analyzed in the present chapter.

In mammals there are three classes of PI3K but so far only the class IA subgroup has been implicated in cancer [12]. They are composed of heterodimers of the p85 family regulatory subunit and the p110 family catalytic subunit (described in more detail in paper VII). They are capable of phosphorylating the inositol ring of the phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP₃). PIP₃ acts by localizing a subset of signalling proteins with pleckstrin homology domains to the membrane (PDK1 and AKT), where they become activated and initiate downstream signalling events [13, 14]. On the other hand RAS, through RAF, activates the dual threonine/tyrosine kinase MEK1/2, which in turn will phosphorylate and activate the ERK1/ERK2 protein kinases. ERK phosphorylates a wide variety of substrates that mediate cell growth and cell cycle entry. In addition to transcription factors (for example MYC, ELK1 and c-FOS), ERK phosphorylates and activates the S6K and the MNK family of kinases [13, 15].

ALTERATIONS OF PI3K PATHWAY COMPONENTS IN BREAST CANCER

The most common genetic alteration of the PI3K signalling pathway found in human cancers, namely breast cancer, is the inactivation of the tumour suppressor gene *PTEN* (an antagonist of PI3K) that leads to the accumulation of PIP₃ [16]. This inactivation can occur through mutations [17], homozygous or heterozygous deletions [18] or even through transcriptional repression and epigenetic silencing due to promoter hypermethylation [4, 5]. The PI3K pathway can also be activated through either gain of function mutations of *PIK3CA* (that codifies one of the PI3K catalytic subunits - p110 α) and of *AKT1* [3, 6], or by gene amplification of *PIK3CA* [7] and of *AKT2* [8]. The mutation rate in invasive breast cancers is between 8-35% for *PIK3CA* and 6% for *AKT1* [3, 19-23] and the amplification rate is 8.7% for *PIK3CA* [7] and 2.3% for *AKT2* [8]. All these alterations are described irrespectively of the molecular subtype of breast carcinomas. Another mechanism of PI3K activation is through RTK signalling, as in cancer these are often mutated, amplified or overexpressed. For example in breast, PI3K can be activated by HER2 (an RTK frequently amplified in breast cancer) [24]. PI3K is also an effector of RAS-mediated oncogenic signalling [25], and several studies have established a role for PI3K activation in the initiation of RAS-driven tumours [26, 27].

ALTERATIONS OF RAS-RAF-MAPK PATHWAY COMPONENTS IN BREAST CANCER

The most common alteration in the MAPK pathway found in human cancer is mutational activation in the *RAS* gene that occurs in approximately 30% of the cases [28]. Although many RAS GTPases have been identified, activating mutations have been reported for only three RAS isoforms: *KRAS*, *HRAS* and *NRAS*, with the alterations restricted to codons 12, 13 and 61 [9]. Activating *KRAS* and *NRAS* mutations are common in malignancies of the pancreas, lung and colon as well as in myeloid leukaemia [29-32], whereas *HRAS* mutations are more frequent in bladder cancer [33]. Activating *BRAF* mutations are encountered in many tumour types, like melanomas, thyroid and colorectal carcinomas, mainly restricted to exons 11 and 15 of the gene [34-36]. Intriguingly in breast cancer, mutations in *RAS* genes are rare [10]. However the importance of the MAPK pathway in breast cancer is evident by its association with increased metastasis risk [37], and to endocrine therapy resistance [38-42]. There are two main mechanisms that might account for endocrine resistance: (1) ERK might phosphorylate Ser¹¹⁸ of ER, which leads to ligand independent activation of ER [38, 43]; or (2) resistance involving this pathway might be acquired through HER2 overexpression [42]. Several studies have shown that tamoxifen therapy increases the expression of both EGFR and HER2 and that these molecules were highly expressed in resistant tumours [38, 42]. Clinical studies have demonstrated that patients whose breast cancers overexpress EGFR and/or HER2 are less likely to benefit from tamoxifen treatment [42]. It is possible that EGFR and HER2 provide survival signals that override apoptotic programs in the cell [44]. Besides endocrine resistance, the MAPK signalling pathway has also been associated with resistance to chemotherapy. Particularly, in triple negative breast cancers, MAPK overexpression was associated with anthracycline resistance and also with increased risk for recurrence [45].

In this chapter we wanted to determine the involvement of the PI3K and RAS-RAF-MAPK signalling pathways in the development/progression of basal-like carcinomas by addressing some of their pathway effectors. Our rationale was based on the knowledge that basal-like breast carcinomas express high levels of EGFR [46-49], and activation of the receptor is known to lead to the activation of both PI3K and RAS-RAF-MAPK. Although in our series EGFR was mainly associated with the subset of metaplastic breast carcinomas, other basal-like gene products besides EGFR, like c-KIT, $\alpha 6\beta 4$ integrin or α B-crystallin are also known to activate both PI3K and RAS-RAF-MAPK [50]. The association of these signalling pathways with basal-like breast carcinomas could also raise new possibilities for therapeutic intervention, as many of these pathway constituents already present target therapies.

MATERIALS AND METHODS

PI3K AND MAPK SIGNALLING EVALUATION IN INVASIVE BREAST CARCINOMAS

The invasive breast carcinoma series (described in Chapter I) was assessed by immunohistochemistry for some of the effectors of both PI3K and RAS-RAF-MAPK. The immunohistochemistry was performed using the Envision Detection System (DakoCytomation, Carpinteria, CA, USA), with the assay conditions described in Paper II. The list of primary antibodies used, class, clone, suppliers, dilutions, time and temperature of incubation as well as antigen retrieval conditions are described in Table 3.1.

Table 3.1 – Characteristics of the primary antibodies used

Primary Antibody	p - residue	Class	Clone	Dilution	Time/Type of incubation	Supplier	Antigen Retrieval
p110 α	-----	RM	C73F8	1:200	ON/4°C	Cell Signaling	AUS
p110 β	-----	MM	ab55593	3 μ g/mL	ON/4°C	Abcam	EDTA
p-AKT	Ser473	RM	736E11	1:50	ON/4°C	Cell Signaling	AUS
p-mTOR	Ser2448	RM	49F9	1:50	ON/4°C	Cell Signaling	AUS
p-NFKB	Ser993	RM	178F3	1:250	ON/4°C	Cell Signaling	EDTA
PTEN	-----	MM	6H11	1:300	ON/4°C	Cascade Bioscience	AUS
pERK1/ERK2	Thr202/Tyr204	RM	20G11	1:100	ON/4°C	Cell Signaling	EDTA

p-residue – phosphorylated residue RM – rabbit monoclonal; MM – mouse monoclonal; ON – overnight; AUS – antigen unmasking solution pH6;

Immunostaining was evaluated as follow:

- **p110 α** and **p-NFKB**: membrane/cytoplasmic expression of p110 α or cytoplasmic expression for p-NFKB was scored as the percentage of labeled cells (ranging from 0 to 100%) multiplied by the intensity of the staining (1- weak, 2-moderate and 3-strong) being a case considered positive whenever the score was above 150;
- **p110 β** : was scored as negative, granular cytoplasmic or membrane staining;
- **PTEN**: cytoplasmic PTEN was scored based on the intensity of the staining obtained in the tumour compared to internal positive controls (blood vessels). Cases were scored as negative (no tumour expression vs. normal expression in the control), reduced (low expression in the tumour vs. normal expression in the control) normal (when the intensity was the same in both tumour and control); nuclear staining was also registered.
- **p-AKT**, **p-mTOR** and **p-ERK1/ERK2**: cytoplasmic/nuclear expression of p-AKT, cytoplasmic p-mTOR and nuclear p-ERK1/ERK2 were scored as either negative or

positive. Furthermore p-ERK1/ERK2 was also evaluated in the stroma as negative and positive.

PIK3CA MUTATIONAL ANALYSIS

PIK3CA mutational status was studied in the invasive breast carcinoma series. Seventy-eight cases were selected from the different subtypes: 29 luminal A, 21 luminal B, 20 HER2-overexpressing and 8 basal-like. The selection criteria were based on material availability as well as percentage of tumour vs. normal tissue presented on the sample. Only cases with more than 90% of tumour were used in order to avoid normal tissue contamination. The DNA was extracted from the formalin-fixed paraffin embedded tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer protocol. PCR-automated sequencing for exons 9 and 20 was performed, as both exons are hot-spots of the *PIK3CA* gene. Exon 20 was divided into two overlapping fragments (exon 20A and B). The primer sequence and product size are described in Table 3.2. PCR reactions were performed using 100-150 ng of DNA, 0.5µM of each primer, 200µM deoxynucleotide triphosphates, 1x PCR buffer and 1U of taq polymerase. The PCR conditions used were as follows: 95°C (5 min) for 1 cycle, 95°C (30 sec), 60°C (45 sec), 72°C (45 sec) for 35 cycles and a final extension step of 72°C (10 min). All primers were designed to have the same annealing temperature.

Table 3.2 – Primer sequences and product size for the two hot spot exons, 9 and 20 of *PIK3CA*

Primer	Sequence (5'→3')	Size (bp)
PIK3CA9-F	GAT TGG TTC TTT CCT GTC TCT G	282
PIK3CA9-R	CTG AGA TCA GCC AAA TTC AGT	
PIK3CA20A-F	TGG GGT AAA GGG AAT CAA AAG	345
PIK3CA20A-R	TAC TCC AAA GCC TCT TGC TC	
PIK3CA20B-F	ACA TTC GAA AGA CCC TAG CC	237
PIK3CA20B-R	CAA TTC CTA TGC AAT CG GTC T	

PCR products were loaded into a 2% agarose gel, purified and sequenced on an ABI Prism® 3100 (Applied Biosystems) automated sequencer using the BigDye® terminator cycle sequencing kit v.3.1 (Applied Biosystems) and the original primers.

CELL LINES, CELL CULTURE AND IMMUNOFLUORESCENCE FOR P110A AND P110B

Paper VI contains a detailed description of the materials and methods used in this section.

STATISTICAL ANALYSIS

The Statview software package was used for all statistical analysis. Correlations between categorical variables were performed using the chi-square test and between continuous and categorical values with analysis of variance. Overall survival (OS) was expressed as the number of months from diagnosis to the occurrence of an event (disease-related death). OS analysis was estimated using Kaplan–Meier method and compared using the log-rank test. In all analysis, a significant level of 5% was considered.

RESULTS

PI3K AND MAPK SIGNALLING EVALUATION IN INVASIVE BREAST CARCINOMAS

The invasive breast carcinomas series was evaluated according to the different expression patterns of effector proteins of both PI3K and MAPK signalling pathways. The analysed effectors of PI3K were: p110 α and p110 β (both catalytic subunits of PI3K), p-AKT, p-mTOR, p-NF κ B as well as the tumour suppressor PTEN. For the MAPK pathway the analysed effector was p-ERK1/ERK2. Figure 3.2 depicts the expression patterns of the studied proteins.

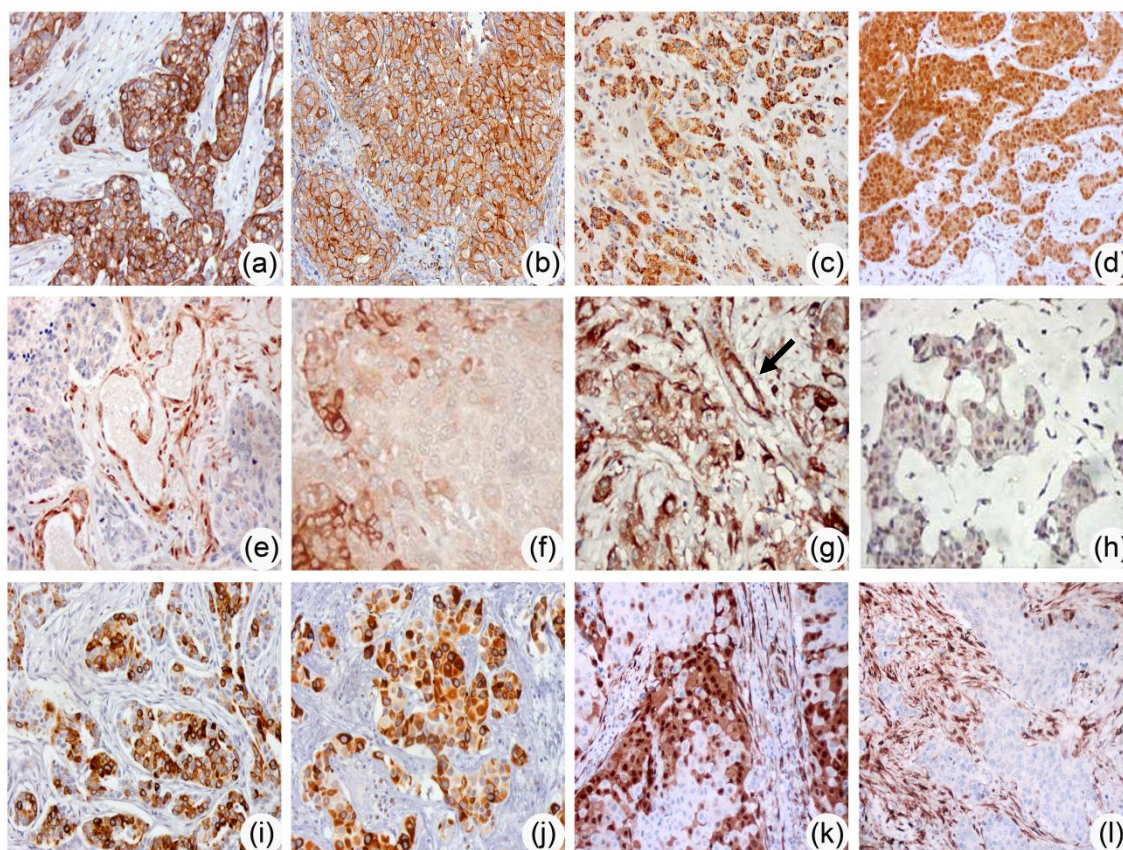


Figure 3.2 – Expression of the PI3K and MAPK signalling effectors in invasive breast carcinomas. (a-j) PI3K and (k-l) MAPK: (a) p110 α membrane/cytoplasmic expression; (b) p110 β membrane expression; (c) p110 β granular

cytoplasmic expression; (d) p-AKT nuclear/cytoplasmic expression; (e) PTEN negative case with a strong staining in a vessel; (f) PTEN reduced with a clear difference in intensity in different areas of the tumour; (g) PTEN normal expression with both tumour and vessel (arrow) stain in the same degree of intensity; (h) PTEN nuclear expression; (i) p-mTOR cytoplasmic expression; (j) p-NFκB cytoplasmic expression; (k) p-ERK1/ERK2 nuclear expression; and (l) p-ERK1/ERK2 stromal expression.

The expression patterns of all these markers were correlated with the different subtypes of invasive breast carcinomas (Table 3.3). p110α was expressed in 11.5% (39/338) of the cases, p110β in 15% (48/320), p-AKT in 71.5% (249/348), p-mTOR in 66.7% (231/348) and p-NFκB in 36.9% (117/317).

Table 3.3 – Associations between the PI3K or MAPK pathway effectors and the subtypes of breast carcinomas

			Subtype				<i>p</i>
			Luminal A (n=256)	Luminal B (n=38)	HER2-overexpressing (n=35)	Basal-like (n=33)	
PI3K signalling	p110α	negative	219 (92.0%)	34 (91.9%)	28 (80.0%)	18 (64.2%)	<0.0001*
		positive	19 (8.0%)	3 (8.1%)	7 (20.0%)	10 (35.7%)	
	p110β	negative	224 (96.5%)	21 (60.0%)	4 (16.0%)	22 (82.1%)	<0.0001*
		granular membranar	6 (2.6%) 2 (0.9%)	4 (11.4%) 10 (28.6%)	1 (4.0%) 20 (80.0%)	5 (17.9%) 0 (0%)	
	p-AKT	negative	77 (31.3%)	8 (22.2%)	4 (11.4%)	10 (32.3%)	NSS
		positive	169 (68.7%)	28 (77.8%)	31 (88.6%)	22 (67.7%)	
MAPK signalling	p-mTOR	negative	69 (28.1%)	12 (32.4%)	11 (32.4%)	25 (80.6%)	<0.0001*
		positive	177 (71.9%)	25 (67.6%)	23 (67.6%)	6 (19.4%)	
	p-NFκB	negative	141 (61.8%)	17 (50.0%)	19 (70.4%)	23 (82.1%)	0.05*
		positive	87 (38.2%)	17 (50.0%)	8 (29.6%)	5 (17.9%)	
	PTEN	negative	94 (37.9%)	10 (27.8%)	4 (11.4%)	20 (64.5%)	<0.0001*
		reduced	72 (29.0%)	11 (30.5%)	22 (62.9%)	9 (29.0%)	
		normal	60 (24.2%)	14 (38.9%)	9 (25.7%)	2 (6.5%)	
		nuclear	22 (8.9%)	1 (2.8%)	0 (0%)	0 (0%)	
	p-ERK1/ERK2 tumour	negative	116 (47.5%)	26 (72.2%)	23 (69.7%)	25 (80.6%)	0.0001
		positive	128 (52.5%)	10 (27.8%)	10 (30.3%)	6 (19.4%)	
	p-ERK1/ERK2 stroma	negative	132 (53.4%)	17 (47.2%)	11 (33.3%)	17 (53.1%)	NSS
		positive	115 (46.6%)	19 (52.8%)	22 (66.7%)	15 (46.9%)	

* statistically significant; NSS – not statistically significant

When analysing the canonical PI3K pathway (Figure 3.3a - p110 α or β /p-AKT and either p-mTOR or p-NF κ B) it was possible to observe that from the 39 cases with p110 α expression, 34 also presented AKT activation, of which 22 had p-mTOR. As for p110 β , of the 48 positive cases, 39 were p-AKT positive, of which 25 were p-mTOR positive. p-NF κ B was not an alternative pathway to p-mTOR as all the cases (either activated via p110 α or p110 β) that were p-NF κ B positive were also positive for p-mTOR. Although the canonical pathway was not represented in the majority of the cases, p-AKT can also be activated through other pathways, leading also to the activation of the other downstream targets p-mTOR and p-NF κ B (Figure 3.3b). Also p-mTOR and p-NF κ B can be activated by alternative PI3K effectors rather than AKT (Figure 3.3c). This may account for the higher expression of these proteins as compared to the p110 isoforms.

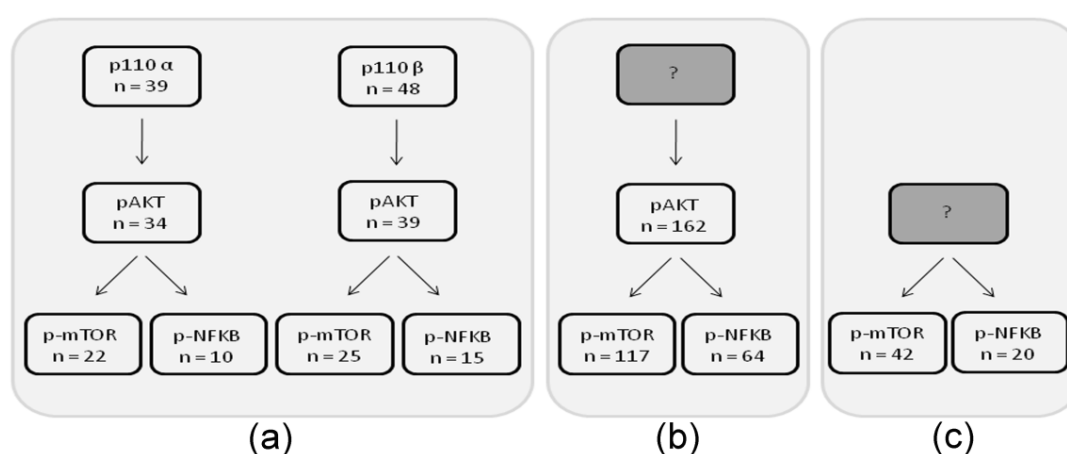


Figure 3.3 – Schematic representation of the PI3K signalling pathway results: (a) canonical pathway where either p110 α or β will lead to AKT activation and subsequent mTOR and NF κ B activation; (b) AKT can be activated by other proteins besides p110 leading to the activation of mTOR and NF κ B; (c) mTOR and NF κ B can also be activated by alternative PI3K effectors, other than AKT.

From the analysis of the tumour suppressor gene PTEN, it was observed that the majority of tumours had either lack (36.6% - 128/350 cases) or reduced (32.6% - 114/350 cases) PTEN expression. Normal PTEN expression was observed in 24.3% of the cases. Interestingly PTEN nuclear expression was observed in 6.6% of the cases (23/350).

The RAS-RAF-MAPK pathway activation, determined by the activation status of its downstream signalling effector p-ERK1/ERK2, was observed in both tumour and stromal cells. In tumours, p-ERK1/ERK2 was expressed in 44.8% of the cases (154/344) whereas stromal expression was observed in 49.1% of the cases (171/348). Concomitant activation of both PI3K and MAPK, resulting from co-expression of both p-AKT and p-ERK1/ERK2, was observed in 37% of the cases (126/336).

When refining the analysis to consider the different subtypes of breast carcinomas it was possible to observe that compared to the other subtypes, basal-like carcinomas presented a higher percentage of p110 α positivity (35.7%). However when considering the other pathway constituents like p-mTOR and p-NF κ B, the majority of basal-like carcinomas were negative for both proteins expression (80.6% and 82.1% respectively). p-AKT was positive throughout all the subtypes, being slightly higher in the HER2-overexpressing (88.6%). Strikingly, the majority of basal-like breast carcinomas (64.5%) lacked PTEN expression. In the other subtypes, PTEN negativity was observed in 37.9% of luminal A, 27.8% of luminal B and 11.4% of HER2-overexpressing. Normal PTEN expression was mainly observed in the luminal subtypes. Reduced PTEN expression was more frequently encountered in HER2-overexpressing cases (62.9%). Another interesting observation was the strong association between the expression of p110 β and the HER2 positive tumours (HER2-overexpressing and luminal B). These results are discussed below.

Noteworthy, the analysis of p-ERK1/ERK2 revealed that this is not a major pathway in basal-like breast carcinomas as the majority of the tumours were negative for ERK activation (80.6%). Expression of p-ERK1/ERK2 was more frequently observed in luminal A tumours (52.5%). Interestingly HER2-overexpression tumours had a higher percentage of p-ERK1/ERK2 expression in the stroma - 66.7%, as opposed to lack of expression in the tumour cells observed in 69.7% of the cases.

p110 β DIFFERENTIAL EXPRESSION IN INVASIVE BREAST CARCINOMAS

The results on the role of p110 β are thoroughly described in Paper VI. In brief, for p110 β two patterns of expression were clearly observed: membrane staining and granular cytoplasmic staining. The membrane expression of p110 β was associated with HER2-overexpression, lower age of onset, higher grade, lymph node involvement, distant metastasis and was inversely associated with HR status. Hence, it was associated with worse prognosis. Furthermore, p110 β expression (either membrane or granular) was associated with worse overall survival. Interestingly the expression of the p110 subunits, α and β , was mutually exclusive as co-expression of both isoforms was only observed in 10 cases. Additionally, after analyzing p110 β in the series of invasive breast carcinomas we assessed its expression in representative breast carcinomas cell lines in order to: (1) determine if the phenotypes observed in the invasive breast carcinomas were also observed and (2) to establish an *in vitro* model for further validation of the results observed. The epithelial cell lines MCF7 (HR positive), BT474 and SKBR3 (both with HER2-overexpression) and the mesenchymal cell line HS578T were selected. All cell lines

expressed p110 α with no differences in cellular localization. Regarding p110 β , MCF7 lacked expression, BT474 presented a membrane/granular cytoplasmic staining, SKBR3 presented a strong membrane expression and HS578T presented a granular cytoplasmic staining.

PIK3CA MUTATIONAL ANALYSIS

From the 78 cases of invasive breast carcinomas analysed, *PIK3CA* mutations in either exons 9 or 20, were observed in 19.2% (15/78 cases). In Figure 3.4 *PIK3CA* functional domains are represented with the corresponding mutations found in the invasive breast carcinomas annotated. Two examples of the chromatograms obtained for the E545K alteration in exon 9 and for the H1047R in exon 20 are also included. All the mutations were already described in the literature.

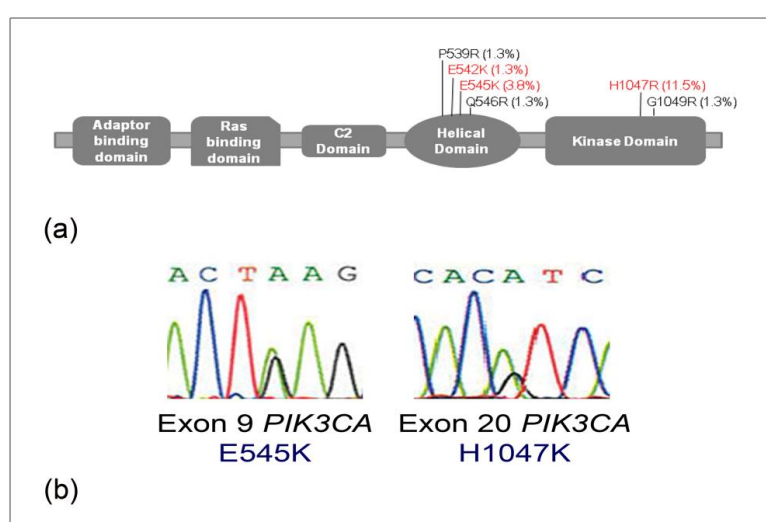


Figure 3.4 – *PIK3CA* mutations encountered in the 78 cases of invasive breast carcinomas analysed (a) *PIK3CA* functional domains with the mutations found in the invasive breast carcinomas annotated; in red the three most common alterations of *PIK3CA* found in cancers are depicted (E542K, E545K, H1047R). The other *PIK3CA* alterations are less common but interestingly cluster near the hot spot regions. H1047R mutation was observed in 9 cases, E545K in 3. E542K and G1049R were each observed in 1 case. Mutations P539R and Q546R were found in the same case (double mutant); (b) example of two chromatograms showing the E545K mutation of exon 9 and the H1047R mutation found in exon 20.

PIK3CA mutation status was then correlated with the different subtypes, as well as with clinicopathological characteristics. The majority of *PIK3CA* mutations were observed in luminal A (9/29) and basal-like carcinomas (3/8). An inverse correlation between HER2 positive tumours and *PIK3CA* mutations was found (from the 41 cases with HER2 positivity only 4 presented *PIK3CA* mutations; $p=0.005$). *PIK3CA* mutations did not correlate with p110 α expression (from the 15 mutated cases, 7 had p110 α expression and 8 did not), nor with overall survival.

DISCUSSION

Altered cell signalling has for long been recognized as a mechanism employed by cells in the development and progression of cancer [51]. PI3K and MAPK signalling pathways are just some of the many signalling modules that have been implicated in disease development. Although few amongst many, both pathways have been recognized as major players in cancer development, as they regulate many important mechanisms such as, cell survival, cell metabolism, cell cycle progression and cell growth [14, 52]. In breast cancer, both pathways have been associated with the development of basal-like carcinomas, either through their association with EGFR or with other basal-like gene products described, like c-KIT, $\alpha 6 \beta 4$ integrin or αB -crystallin [50].

In the present study, the involvement of both pathways in invasive breast carcinomas was assessed by analysing some of the pathways signalling effectors. For PI3K signalling pathway, p110 α and β (PI3K catalytic subunits), p-AKT, p-mTOR and p-NF κ B were analysed. The tumour suppressor PTEN, a counteractant of PI3K pathway activation, was also studied. From the three branches of MAPK that exist in mammalian cells the RAS-RAF-MAPK was chosen, as it is the most frequently associated with breast cancer, not only in basal-like breast carcinomas [2] but also related to development of endocrine resistance [53].

Regarding PI3K signalling pathway there was a statistically significant association between the subtypes of breast carcinomas and both PI3K catalytic subunits (p110 α and p110 β) as well as with the downstream targets, p-mTOR and p-NF κ B. Although the expression of p-AKT was not statistically significant, the high expression of this protein in the invasive carcinomas series indicates a putative role for the activation of this protein in either development or progression of cancer. The PI3K catalytic subunits, p110 α and p110 β , were expressed in around 24% of the cases being p110 α expressed in 12% and p110 β in 15%. According to our results and other publications [54], p110 α was more frequently expressed in basal-like carcinomas. However when considering invasive breast carcinomas as a whole rather than subdividing them into subtypes, p110 α positive tumours are more frequent in hormone receptor positive cases. This level of complexity and of apparent contradictory results, has for long accompanied the studies regarding either the *PIK3CA* gene or its codified protein, p110 α . Although very few studies have addressed p110 α 's expression, the *PIK3CA* gene has been target of much scrutiny in these last years, due to the high mutation rate encountered [55]. In breast cancers, *PIK3CA* mutations are a common event but as previously stated their correlation with prognosis is still debatable, since some authors find them to be associated with hormone receptor positivity [19, 21, 23, 56-58], while others are unable to determine such association [20, 59-61]. Also, the association between

presence of mutations in *PIK3CA* and HER2 is also unclear. There are findings that show a correlation between HER2 overexpression and *PIK3CA* mutations [19, 22, 58], whereas some report the exact opposite (*PIK3CA* mutations associated with HER2 negativity) [23, 57], while others were not able to find any association between both variables [20, 56, 60]. These inconsistencies are also present when associating *PIK3CA* mutations and other clinicopathological variables like age, histological type, tumour grade, stage and presence of lymph node metastasis [19-21, 23, 56-61]. *PIK3CA* mutations were also investigated in the invasive breast carcinoma series. The selection criterion was based on tissue availability as well as amount of tumour present in the sample: only cases which paraffin blocks presented more than 90% of tumour (as observed in the H&E staining) were considered, in order to avoid contamination by normal tissue. Another criterion was the enrichment in HER2 positive samples as, at the time of the first analysis, the paper by Saal *et al* [19] had just been published, claiming the high mutation rate encountered in HER2 positive tumours, irrespectively of ER status. Using the above criteria, 78 cases were selected and the described hotspot regions of the gene, exons 9 and 20, which codify the helical domain and the kinase domain, respectively, were analysed. The mutation frequency encountered was 19.2% (15 out of 78 cases) which is in agreement with the literature that ranges from 8 to 35% [3, 19-23]. The majority of the mutations occurred in exon 20 which is in accordance with other published data, showing that in breast this is the most frequently mutated exon [19]. The most frequent mutation was the H1047R (corresponding to 9 of the 15 mutated cases). One case presented a double mutant with the concomitant occurrence of both P539R and Q546R. The significance of this double mutant is unknown but might simply reflect the idea that some mutations are less potent and need an additional alteration to confer advantage. *PIK3CA* mutations were then correlated with the different subtypes as well as with the patients and tumours characteristics. *PIK3CA* mutations were more frequent in luminal A cases (9/29) and basal-like carcinomas (3/8). Interestingly one of the basal-like breast carcinoma cases that presented a mutation was a metaplastic carcinoma; a histological type that recently has been shown to present a high mutation rate of *PIK3CA* [62]. *PIK3CA* mutations were not related with HER2-overexpression as only 4 out of 41 cases were mutated. Noteworthy, 3 were luminal B, therefore also presenting ER positivity. Surprisingly, the presence of mutations in *PIK3CA* gene did not correlate with p110 α expression. This discrepancy in results can be added to the huge amount of contradictory published data related with *PIK3CA* and p110 α , and confirms the need for validation of the actual biological significance of these players in large cohorts of patients, making use of consensual tools in both assessment and evaluation procedures. Recently the largest analysis of *PIK3CA* mutations in breast cancer was conducted in a cohort of 590 patients

with a median follow up of 12.8 years. It was demonstrated that *PIK3CA* mutations were associated with favorable clinicopathological features like, older age, hormone receptor positivity, HER2 negativity, lower tumor grade, stage and lymph node negativity. Patients with *PIK3CA* mutations presented a better clinical outcome, including survival [23]. In other cancer models like colorectal, *PIK3CA* mutations occurred more frequently associated with *KRAS* or *BRAF* mutations rather than isolated, suggesting a possible synergistic effect [63] which might be seen as a need for more oncogenic potency. Recently, Dan *et al* [64] demonstrated that the response to PI3K inhibitors was not dependent on *PIK3CA* mutational status. The *PIK3CA*-mutant cell lines were susceptible to PI3K inhibitors to a similar extent as the wild type *PIK3CA*.

In contrast to *PIK3CA*, no cancer specific mutations have been reported in the gene encoding the other catalytic subunit p110 β . Only recently the link between p110 β and cancer was established with several pioneer studies. In an animal model, of prostate tumor formation induced by *PTEN* loss, the ablation of p110 β , but not of p110 α , was sufficient to block tumorigenesis [65]. This link of p110 β and *PTEN* was also observed in a colorectal cancer model, where in *PTEN*-negative cancers, p110 β , instead of p110 α , proved to be the essential isoform for signaling and replication [66]. In breast, mice expressing a catalytic inactive form of p110 β were protected from tumor development, in a HER2-driven cancer model. In human breast cancer samples it was observed that copy number gain of *PIK3CB* occurs at a low frequency (approximately 5%) but might present clinical significance [67]. The results published in Paper VI were the first to report the expression of p110 β in invasive breast carcinomas. The putative relationship between p110 β and breast cancer was highlighted by its association with lower age of onset of the disease, higher tumor grade, lymph node involvement and presence of metastasis in distant organs. In addition p110 β inversely correlated with hormone receptor status and presented a striking association with HER2 positive tumors, being expressed in 93.8% of these cases. This pattern of aggressiveness was also reflected by its association with worse overall survival. Furthermore, all the cell lines studied that presented HER2-overexpression (SKBR3 and BT474), also presented high p110 β expression. Apart from membrane expression, p110 β also presented a granular pattern of expression. The biological significance of the granular expression is still unclear and requires further studies. Still, its association with worse overall survival suggests that this was not due to a staining artefact, but possibly p110 β is engaged in other cellular functions. It was already described that p110 β can also present a kinase-independent function with an important role in RTKs endocytosis [68].

Activation of AKT was observed in the majority of the cases, not only those with p110 expression (thus not following the canonical PI3K pathway), a fact that is explained by the integrating role

that AKT has in cells, as it is able to be activated through a huge number of other signalling networks, like RAS and SRC [69]. As for AKT downstream targets neither p-mTOR nor p-NFκB were major players in basal-like breast carcinomas as most of these tumours were negative for the expression of both activated forms. p-mTOR was highly expressed in all the other subtypes whereas p-NFκB was more prominently expressed in luminal B. The expression of the tumour suppressor PTEN was also evaluated, as the association between breast cancer and PTEN has been demonstrated for a long time, not only through the high mutation rate encountered (between 30-50%) [70] or the high percentage of promoter methylation encountered in breast cancers [4], but also related to the usefulness of PTEN as a predictive marker of response to trastuzumab [71, 72]. More recently the lack of PTEN has been associated with basal-like breast carcinomas [73]. For the immunohistochemical analysis of PTEN the clone 6H2.1 was selected as it is the only antibody to show a correlation with the presence of molecular alterations of PTEN [74]. PTEN was evaluated based on 4 observed phenotypes as: negative, reduced, normal and nuclear. PTEN was negative in 36.6% of the cases (128/350). However it was in basal-like breast carcinomas that this absence of expression was more pronounced as it occurred in 64.5% of the cases. This suggests that from all the studied alterations, PTEN loss might be an important driver for tumour development/progression of basal-like carcinomas. Also it might explain the lack of response observed in the clinical trials with anti-EGFR drugs. In one study, conducted in patients with triple negative metastatic disease, where the monoclonal antibody cetuximab was added to carboplatin, EGFR inactivation was not sufficient for a clinical response as the majority of the tumours failed to demonstrate downstream pathways inactivation [75]. Studies in archival tissues from these cetuximab trials suggested that downstream molecules could create a ligand-independent activation state. Preliminary results from this analysis pointed, amongst others, for the benefit of cetuximab treatment in the presence of higher PTEN expression [76]. In this analysed series of invasive breast carcinomas PTEN loss and mutations in the *PIK3CA* gene were not mutually exclusive. This is in agreement with other reports that show coexistence between *PTEN* aberrations and presence of *PIK3CA* mutations [26]. However there are also some opposing studies demonstrating that *PTEN* aberrations and *PIK3CA* mutations are mutually exclusive [9, 19]. The exact outcome of these events remains unsolved. Even if ultimately both alterations lead to PI3K activation, the alterations encountered will probably influence differently tumour development or progression, conferring different selective advantages depending on their capability to induce, cell growth and survival. This may be exemplified by the following observations: both *PIK3CA* mutations and *PTEN* loss have been associated to HER2-targeted therapy [71, 72, 77]; *PIK3CA* mutations are observed in cancers with low PTEN expression, but

this is not related to increased levels of p-AKT [58]; *PTEN* loss is associated with worse prognosis [78] whereas, as previously mentioned, *PIK3CA* mutations effect on clinical outcome is still debatable. All these evidences support the idea of biological differences between *PIK3CA* mutations and *PTEN* alterations in breast tumours.

Interestingly, in the present work PTEN nuclear expression was also observed. This expression pattern was restricted to the luminal subtype. Recently two seminal papers provide a possible role for nuclear PTEN. In response to DNA damage PTEN forms a complex, in the nucleus, with p300 (a histone acetyltransferase that regulates transcription via chromatin remodelling and is important in the processes of cell proliferation and differentiation [79]) maintaining high p53 acetylation [80]. Also, nuclear PTEN was described as having a role in the control of chromosomal integrity. PTEN was found localized at centromeres and was physically associated with CENP-C, an integral component of the kinetochore. It was further shown to act on chromatin and regulating the expression of RAD51, reducing the incidence of spontaneous double strand breaks (DSBs) [81]. This association of PTEN and DSBs is quite interesting as basal-like breast carcinomas are associated with defects in the ability of repairing DSBs. This might account for the high percentage of PTEN negative cases observed in basal-like tumours. Interestingly, Saal *et al* [82] showed that, in mice, inactivation of *Pten* leads to the formation of basal-like tumours, and that loss of PTEN expression is significantly associated with this subtype, both in sporadic and *BRCA1*-associated tumours. Since *BRCA1* is important in DSB repair these authors proposed that *PTEN* alterations might be a cause of *BRCA1* dysfunction as frequent gross *PTEN* mutations, involving intragenic chromosome breaks, inversions, deletions and micro copy number aberrations, occur specifically in *BRCA1*-deficient tumours [82].

Regarding MAPK activation, determined by the phosphorylation status of the complex ERK1/ERK2, it was observed that it was a more common event in luminal A tumours (52.5%) as compared with the other subtypes. Unfortunately, in the studied series of invasive breast carcinomas, there is no information regarding therapy nor patient response as it would be interesting to determine if the activation of this pathway in luminal tumours could somewhat preclude the development of resistance to endocrine therapy [38-42]. Recently, in a systems-based approach used to identify susceptibility to MEK inhibitors, basal-like cell lines were shown to be more prone to respond to MEK-ERK inhibition [2]. In response prediction, CK5 and EGFR were associated with sensitivity to the inhibitor whereas ER and CK18 were associated with resistance [2]. However in the present series, activation of ERK proteins in basal-like carcinomas was only observed in 19.4% of the cases and no association between CK5 or EGFR and p-ERK1/ERK2 was observed. Although not statistically significant, the stromal expression of p-

ERK1/ERK2 was an intriguing result, as it reinforces the idea that the stroma is an integral part of the tumour being able to contribute to the acquirement of a more aggressive phenotype. For long, the majority of cancer research has been focused on the events occurring in the tumour itself. Although this resulted in a revolutionary change in the understanding of altered molecular pathways it neglected the influence of stromal-epithelial interactions. It is accepted that the progression of a lesion to a carcinoma is dependent on the engagement of “reactive stroma” that provides both structural and vascular support for tumour growth, leading also to tissue reorganization and invasiveness [83]. The activation of stromal p-ERK1/ERK2 (highly observed in the HER2-overexpressing tumours – 66.7%) could simply reflect the development of highly tumorigenic and invasive reactive stromal cells representative of possible epithelial-stromal interactions.

Most importantly the data presented demonstrates that breast cancer is able to make use of many signalling cues that regulate important mechanisms like survival and proliferation. In order to maximize the likelihood of understanding its heterogeneity, the exact identity of the signalling networks, their constituents, either activators or repressors, need to be assessed. One possibility of achieving this milestone is using a systems biology approach. The available data from high throughput technologies can be integrated in order to predict how the different components of the network interact and govern the system. This offers the opportunity not only of going one step further on disease knowledge but also enables the identification of other possible therapeutic targets.

KEY FINDINGS

- Lack of PTEN expression was a frequent event in basal-like breast carcinomas, where it is observed in 64.5% of the cases;
- p110 β appeared as a new target in breast cancer, particularly in HER2-positive cases, as it was expressed in 93.8% of this cases;
- AKT was activated in the majority of breast carcinomas, accounting for its integrating role in cells;
- pmTOR and pNF κ B were not major players in basal-like breast carcinomas as most cases did not express these proteins;
- MAPK activation was mainly observed in luminal A tumours (52.5%) as opposed to basal-like breast carcinomas (19.4%).

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CHAPTER 4 – GROWTH FACTOR CIRCUITRY:

EGFR AS A MODEL OF NETWORK DYNAMICS

PAPERS RELATED TO THIS CHAPTER:

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INTRODUCTION

Cells respond to external stimuli using a limited number of signalling pathways that can be activated by plasma membrane receptors, such as GPCR and RTKs. Signalling pathways do not simply transmit but rather engage the multitasking role of processing, encoding and integrating internal and external signals [1]. This shows their pivotal role in cellular decisions that are dependent both on temporal and spatial distribution of the activated signal transducers. For example, RTK-mediated signalling has for long been associated with regulation of embryogenesis, cell survival, motility, proliferation, differentiation, glucose metabolism and apoptosis. As expected, malfunction of RTKs is a leading cause of numerous diseases, including cancer [2, 3]. Upon stimulation, RTKs undergo dimerization (e.g. EGFR) or allosteric transitions (e.g. insulin receptor - IR) that result in the activation of intrinsic tyrosine kinase activity [4, 5]. Subsequent phosphorylation of multiple tyrosine residues on the receptors will transmit a signal to several cytoplasmic proteins which, in turn, will lead to their recruitment to the cell surface [4]. These interactions will result in activation of complex circuits that will ultimately lead to cellular responses. Looking at a more mechanistic level, this network circuitry is regulated by positive and negative feedback loops. Positive feedback occurs when a signal induces more of itself, or of another molecule that amplifies the initial signal, by stabilizing, amplifying or prolonging it. On the other hand, negative feedbacks occur when, for example, a signal induces the expression of its own inhibitor, serving to dampen and/or limiting signalling [6]. The EGFR system is one of the best studied signalling networks [7], being its deregulated activation implicated in various types of human cancer, including breast cancer [8]. The dynamics of signal transduction events induced by EGF at the level of phosphorylation and gene expression can depict the above mentioned concept of positive and negative feedback loops. This concept of signalling network has long replaced the earlier concepts of linear pathways.

POSITIVE FEEDBACK LOOPS

Positive feedback loops enhance the amplitude and prolong the active state of signalling pathways. In the case of the HER family, binding of the ligand to its primary receptor, is tuned by the identity of the dimerization partner. HER2 is considered to be an important positive regulator. It functions as a preferred partner for dimerization [7] and HER2 containing dimers are able to evade negative regulation, as they can move ligand-activated receptors to recycling thus escaping degradation [9, 10]. This will prolong the output signal. Other positive feedbacks are based on autocrine and paracrine loops, in which EGF or EGF-like ligands are produced following

receptor activation [7]. The cellular response to stimulation involves the induction of forward-signalling events that in a normal situation will be counteracted by attenuation mechanisms. However network robustness, that is the ability to maintain performance in the face of perturbations [11], can be exploited in a disease context through persistency of these feed forward signals.

NEGATIVE FEEDBACK LOOPS

Multiple molecular mechanisms, including post-translational modifications, compartmentalization, catalytic inactivation and steric hindrance, participate in signal attenuation. These negative regulators can either pre-exist or can be newly synthesized following activation of the HER family members. Pre-existing attenuators primarily control receptor dephosphorylation and degradation [7]. By acting at the receptor level, tyrosine phosphatases like DEP1, which dephosphorylates EGFR [12], and PTP1B, which dephosphorylates RTKs in the endosomes [13], are able to diminish signal. However receptor internalization coupled to degradation is considered the most effective, irreversible process that attenuates signalling by targeting surface receptors for degradation in lysosomes [14]. As opposed to pre-existing attenuators, the expression level of newly synthesized attenuators rises after stimulation, reaching a peak within an hour of the initial stimulation, thereby defining the window of active signalling. For example EGF treatment induces the expression of SOCS5 that will lead to the degradation of EGFR [15].

In biology these feedbacks, either positive or negative, always carry the risk of signalling control loss, and subsequent breakdown of homeostasis that ultimately lead to disease. These interplays between forward signalling and feedback regulation were elegantly demonstrated by Amit *et al* [16] where the authors addressed the dynamics of growth factor signalling. They demonstrated, through profiling of protein phosphorylation and gene expression, the presence of a kinetically defined cluster of delayed early genes (DEGs) that function to attenuate the early events of growth factor signalling. Using EGFR as a model system it was observed that DEG cluster functioned as a feedback regulator of immediate early genes. This was consistent with the downregulation of DEG cluster in diverse tumour types, determined by the analysis of the human cancer compendium that comprise 1975 published microarrays spanning 22 tumour types. As a model, the authors used HeLa cells (a common model for growth factor signalling research studies) stimulated with EGF. The findings in this cell line were then compared to an immortalized breast cancer cell line, MCF10A, stimulated either with EGF or with serum. Interestingly this comparison revealed that the clusters of both immediate early genes and of DEGs were

conserved across the three transcriptional profiles although, only the identity of the genes observed in each cluster differed significantly. This led to the hypothesis, that it was the particular combination of induced genes that defined the identity of the active cellular pathway, as already suggested by other authors [17].

This system knowledge of signalling networks will allow the identification of the underlying mechanisms that govern them and that might be targeted for pharmacological intervention. In order to refine the knowledge on EGFR network dynamics, we characterized outputs of positive regulation and a negative regulator of this signalling pathway. The EGF-induced, C-terminal tensin like domain (CTEN) and Synaptojanin2 (SYNJ2), were initially identified in the analysis of a data set that used EGFR as a model system to study signalling networks [16]. The human lateral signaling target protein 2 (LST2), was studied in the context of negative regulation of EGFR. All were thus assessed in the invasive breast carcinoma series described in Chapter 1.

MATERIALS AND METHODS

C-TERMINAL TENSIN LIKE DOMAIN (CTEN)

Paper VIII contains a detailed description for the CTEN analysis.

The identity of EGF-induced genes was assessed using the transcriptional profiling data set from the EGF-treated human HeLa cells, described by Amit *et al* [16]. Analysis of this data set revealed that prolonged EGF treatment induced the expression of various genes that are involved in the regulation of cell adhesion or organization of the actin cytoskeleton. One of these genes was CTEN.

For the immunohistochemistry analysis of CTEN, an anti-CTEN antibody was generated by immunizing mice with a GST-cten fusion protein (amino acids 181-353). Immunohistochemistry for CTEN was performed using the Envision Detection System. The assay conditions are described in Paper VIII. Briefly, the antigen retrieval was EDTA pH9, at 98°C for 20 minutes and the primary antibody was diluted 1/40 (initial concentration of the antibody 0.3 mg/mL) incubated ON at 4°C.

SYNAPTOJANIN2 (SYNJ2)

Genes that were observed to be upregulated only by EGF treatment, and not by serum, in MCF10A [16] were compared to the expression profile of a highly metastatic breast cancer cell line, MDA-MB-231LM2 [18]. This comparison enabled the identification of common upregulated

genes in both cell lines that could be important for cell migration and metastization (EGF dependent). One of the identified genes was SYNJ2.

SYNJ2 was then studied in *in vitro* models where it was associated with increased migration and invasiveness potential. Furthermore in a mouse model SYNJ2 was shown to be related with metastatic capacities.

For the immunohistochemical assessment of SYNJ2 tissue sections were deparaffinised followed by antigen-retrieval with a commercially available EDTA solution, pH9 at 1:10 at high temperature (water bath, 20 minutes at 98°C). The endogenous peroxidase activity was blocked using a commercially available solution. The slides were then incubated with the primary antibody, SYNJ2 mouse monoclonal clone 2H8 (Abnova, Taiwan), 1/100 (initial concentration of the antibody 1mg/mL) for 75 minutes at room temperature. The Envision Detection System was used for the visualization of the reaction with diaminobenzidine as chromogen. Tissues were then counterstained with Mayer's haematoxylin, dehydrated and coverslipped.

LATERAL SIGNALING TARGET PROTEIN 2 (LST2)

Paper IX contains a detailed description for the LST2 analysis.

Immunohistochemistry for LST2 was performed using the Envision Detection System as previously referred for CTEN and SYNJ2. Assay conditions are the same as the ones described in Paper IX. Briefly the slides were pre-treated with citrate buffer pH6 in the PT Module (Lab Vision, Fremont, CA, USA), at 98°C for 30 minutes. Anti-hLst2 antibody (clone 398) was applied at 1/500 dilution and incubated for 1h 30m at room temperature.

IMMUNOHISTOCHEMICAL EVALUATION

All the analysed antibodies presented a cytoplasmic expression. However SYNJ2 sometimes was observed in the plasma membrane. CTEN and LST2 were evaluated as either negative or positive and SYNJ2 was semi-quantitatively scored as 0: negative; 1+: weak staining or 2+: strong staining.

STATISTICAL ANALYSIS

The Statview software package was used for all statistical analysis. Correlations between categorical variables were performed using the chi-square test and the Spearman's correlation rank. In all analysis, a significant level of 5% was considered.

RESULTS

C-TERMINAL TENSIN LIKE DOMAIN (CTEN)

Paper VIII contains a detailed description of the CTEN results. Hence only a brief description of the key findings will be mentioned.

From the analysis of the data set of HeLa cells treated with EGF an alteration in the expression of two members of the tensin family: Tensin 3 (TNS3) and CTEN was observed. This family comprises four proteins (Tensin 1, 2, 3 and CTEN), which are involved in cell migration, and are localized to focal adhesion sites. All four tensin family members contain a SH2 and a PTB domain in their carboxyl termini. Through the PTB domain, tensins are able to bind β -integrins, which are transmembrane cell-adhesion molecules. Tensins 1, 2 and 3 contain an actin binding domain in their amino termini, while CTEN is a shorter protein that lacks this domain. Following EGF-treatment TNS3 mRNA undergoes downregulation, whereas CTEN expression exhibits upregulation. Similarly to the observation made in HeLa cells, treatment of MCF10A cells with EGF resulted in induction of CTEN and reduction in TNS3 levels. It was observed that the induction of CTEN was a result of ERK/MAPK activation as the MEK inhibitor (U0216) completely blocked CTEN induction. Following EGF-treatment actin-stress fibres, which are normally anchored to paxillin-containing focal adhesion sites, disintegrate and reorganize in cortical bundles. Additionally, using short interfering RNA (siRNA) techniques it was possible to observe that upon depletion of CTEN, EGF-stimulated cells presented large, densely packed colonies that resembled the morphology of cells grown in the absence of EGF. Also CTEN depleted cells presented a restoration of a dense network of actin fibres, even in the presence of EGF in the growth medium, and a 70% reduction in cell migration. As for TNS3 depletion, actin stress fibres disintegration was observed even in the absence of EGF. In addition, under these conditions, TNS3 depletion was sufficient to promote partial cell migration. Taking all these results into account and the knowledge that HER family of receptors (particularly EGFR and HER2) are highly involved in the development of breast cancer, CTEN expression was analysed in a series of 272 cases of invasive breast carcinoma. The results are summarized in Table 1 of Paper VIII. A statistically significant correlation was observed between CTEN expression and histological grade ($p=0.001$), lymph node involvement ($p=0.001$), ER ($p=0.004$), HER2 ($p=0.004$) and EGFR ($p=0.0006$). No correlation was observed between CTEN expression and tumour size. CTEN positive cases had high expression of EGFR and HER2 and were inversely correlated with ER

expression. They had higher tumour grade and the ability of metastasizing to the lymph nodes. Figure 4.1 depicts an example of CTEN's expression in an invasive breast carcinoma.

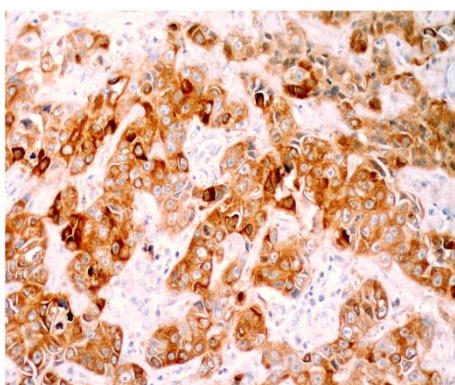


Figure 4.1 – CTEN expression in an invasive breast carcinoma tumour sample

CTEN was more frequently observed in basal-like tumours (Table 4.1). Interestingly, the two other subtypes with worse prognosis, luminal B and HER2-overexpressing tumours also present a high expression of CTEN, 34.3% and 31.4% respectively. In the luminal A group CTEN was only expressed in 5.9% of the cases.

Table 4.1 – CTEN expression in the subtypes of breast carcinomas

Characteristic		CTEN		<i>p</i>
		Positive	Negative	
Subtype	Luminal A	15 (5.9%)	238 (94.1%)	<0.0001
	Luminal B	12 (34.3%)	23 (65.7%)	
	HER2-overexpressing	11 (31.4%)	24 (68.6%)	
	Basal-like	12 (37.5%)	20 (62.5%)	

Furthermore in an analysis of a cohort of patients with inflammatory breast carcinomas, CTEN expression was detected in 56% of the tumour biopsies. These tumours present expression of both HER2 and phospho-EGFR for which there was an interest in testing the effect of the dual inhibitor lapatinib. Immunohistochemical analysis of the biopsies before and after treatment revealed a statistically significant downregulation of CTEN, following lapatinib treatment, in the majority of the tumours.

SYNAPTOJANIN2 (SYNJ2)

Synaptojanins (SYNJs) are a family of phosphatidylinositol (PI) phosphatases that contain two distinct phosphatase domains arranged in tandem: a SacI homology domain that acts on PI3P, PI4P, and PI3,5P₂ and an inositol 5'-phosphatase domain, acting both on PI4,5P₂ and PI3,4,5P₃ [19, 20]. Up to now, two SYNJs were described in the human genome: the major splice isoform of

SYNJ1 that is brain specific and the ubiquitously expressed SYNJ2 [21, 22]. SYNJ2 functions in clathrin-mediated endocytosis [23] and has been localized to various cell compartments, including the outer mitochondrial layer [24] and the plasma membrane [25]. Amongst other functions SYNJ2 has been recently implicated in the regulation of cytoskeleton, in lamellipodia formation, cell migration and invasion [20, 25].

Preliminary data demonstrated that SYNJ2 was upregulated in EGF-treated MCF10A cells, and its importance in migration and metastasis was assessed both *in vitro* and *in vivo*. In order to determine SYNJ2 involvement in breast cancer progression, the invasive breast carcinomas series was studied for the expression of this protein.

From the analysis of SYNJ2 expression with clinicopathological parameters (Table 4.2) a statistically significant correlation was observed with the different subtypes of breast carcinomas ($p < 0.0001$), proliferation index as assessed by Ki67 ($p = 0.02$), ER ($p < 0.0001$) and HER2 expression ($p = 0.0002$).

Table 4.2 – Association between SYNJ2 expression and clinicopathological parameters in invasive breast carcinomas

Characteristic		SYNJ2			<i>p</i>
		0	1+	2+	
Subtype	Luminal A	77 (32.6%)	87 (36.9%)	72 (30.5%)	<0.0001*
	Luminal B	4 (11.8%)	19 (55.9%)	11 (32.3%)	
	HER2-overexpressing	0 (0%)	8 (27.6%)	21 (72.4%)	
	Basal-like	6 (20.0%)	9 (30.0%)	15 (50.0%)	
Size (cm)	≤2	42 (25.8%)	62 (38.0%)	59 (36.2%)	NSS
	>2	45 (27.2%)	60 (36.4%)	60 (36.4%)	
Grade	I	23 (31.1%)	30 (40.5%)	21 (28.4%)	NSS
	II	42 (30.9%)	48 (35.3%)	46 (33.8%)	
	III	22 (18.8%)	45 (38.5%)	50 (42.7%)	
LN involv.	None	28 (25.9%)	38 (35.2%)	42 (38.9%)	NSS
	Between 1 and 3	19 (23.8%)	32 (40.0%)	29 (36.2%)	
	More than 3	26 (36.6%)	26 (36.6%)	19 (26.8%)	
Metastasis	None	71 (27.0%)	97 (36.9%)	95 (36.1%)	NSS
	Locoregional	3 (25.0%)	4 (33.3%)	5 (41.7%)	
	Distant	10 (23.8%)	18 (42.9%)	14 (33.3%)	
	Both (locoregional and distant)	3 (25.0%)	4 (33.3%)	5 (41.7%)	
Ki67	Low proliferation	55 (28.3%)	77 (39.7%)	62 (32.0%)	0.02*
	High proliferation	21 (19.8%)	34 (32.1%)	51 (48.1%)	
ER	Positive	81 (30.0%)	106 (39.3%)	83 (30.7%)	<0.0001*
	Negative	6 (10.2%)	17 (28.8%)	36 (61.0%)	
HER2	Positive	4 (6.3%)	27 (42.9%)	32 (50.8%)	0.0002*
	Negative	83 (31.2%)	96 (36.1%)	87 (32.7%)	

LN involv – lymph node involvement; NSS – not statistically significant * statistically significant.

High SYNJ2 expression was mainly observed in basal-like carcinomas and HER2-overexpressing group, 50.0% and 72.4% respectively. Interestingly the cell distribution of SYNJ2 was different in basal-like and HER2-overexpressing groups. Figure 4.2 depicts the patterns of expression observed for SYNJ2 in both basal-like and HER-overexpressing tumours. In basal-like tumours SYNJ2 staining was observed in the cytoplasm whereas in HER2-overexpressing tumours it was mostly observed in the plasma membrane.

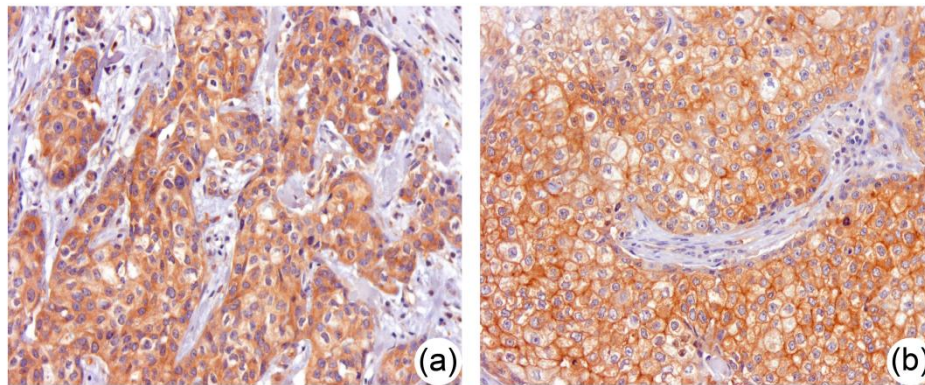


Figure 4.2 – SYNJ2 expression in (a) a basal-like tumour and (b) a HER2-overexpressing tumour.

The vast majority of ER negative cases presented a high expression of SYNJ2. Almost all HER2-positive cases presented SYNJ2 expression. Although not statistically significant, high expression of SYNJ2 was more frequently observed in cases that presented distant metastasis.

LATERAL SIGNALING TARGET PROTEIN 2 (LST2)

Paper IX contains a detailed description for the LST2 results. Hence only a brief description of the key findings will be mentioned.

Endocytosis involves the cellular uptake of extracellular compounds by the invagination and pinch off of defined areas of the plasma membrane. The endocytic vesicles formed will then fuse with early endosomes. From here the endocytosed material can be relocated to a number of alternative destinations [26], either for recycling back to the plasma membrane or for lysosomal destruction at late endosomal compartments [27]. Multiple adaptors engage these endocytic tasks, and many contain a PI3P-binding domain called FYVE. FYVE proteins are mainly involved in endocytotic transport and regulate fusion of endosomal membranes with other endocytic vesicles and organelles [28]. In this work a mammalian FYVE domain protein, termed hLST2,

was described. In brief, it was observed that cellular depletion of hLST2 increased EGF-induced signal. This observation was consistent with the described role of its primitive ortholog in *C. elegans* that functions as a negative regulator of the worm's EGFR [29]. However, despite its intrinsic FYVE domain, hLST2 displayed primarily nonendosomal localization. Through the identification of a specific lysine acceptor, monoubiquitinylation was identified as a means of preventing the association of the FYVE domain of hLST2 to PI3P-enriched endosomes. The removal of monoubiquitin from hLST2 was sufficient for the activation of its cargo sorting activity and blockage of growth factor signalling.

Given its negative role in EGF signalling, the status of hLST2 in human tumours raised interest. For this purpose the series of invasive breast carcinomas was studied, for hLST2 expression. Figure 4.3 shows LST2 staining in an invasive breast carcinoma.

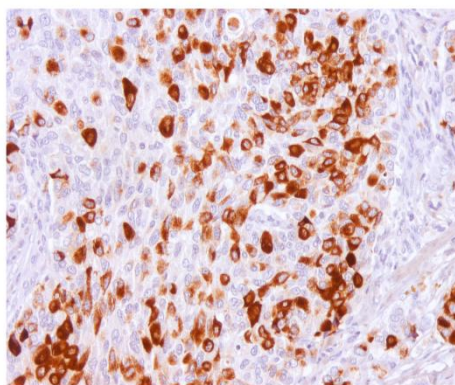


Figure 4.3 – LST2 expression in an invasive breast carcinoma

Of 272 breast carcinomas only 12 cases presented LST2 expression (4.4%). hLST2 positive cases were more frequently observed in luminal A tumours. The tumours were more differentiated and displayed limited metastasis to lymph nodes (Table S1 of paper IX).

DISCUSSION

Cells operate based on information about external conditions, internal processes and upon information stored on their genome [30]. The introduction of large-scale high throughput technologies enabled the prediction of integrating signalling networks and their outcomes [31]. This system-level perspective provides a useful framework that accommodates the increasingly amount of experimental data [7] as opposed to the models based on an oversimplified view of gene expression as simple responses to a determined transcription factor. In the present chapter

the expression of CTEN, SYNJ2 and LST2 was analysed in a series of invasive breast carcinomas. Both CTEN and SYNJ2 were initially identified in the analysis of a data set that used EGFR as a model system to study signalling networks [16]. LST2 was studied in the context of negative regulation of EGFR.

In Paper VIII it was observed that following EGF treatment in HeLa cells a reciprocal alteration in two tensins occurred. TNS3 mRNA underwent downregulation, whereas CTEN's was upregulated. Similar observations were made in the immortalized mammary cell line MCF10A. As migration of MCF10A is reported to be ERK/MAPK-driven, the MEK inhibitor (U0126) was used to determine if this could be dependent on the induction of regulatory proteins like CTEN. The inhibitor completely blocked CTEN induction.

EGF-induced upregulation of CTEN and downregulation of TNS3 was correlated with actin fibres remodelling, as upon EGF-stimulation the actin stress fibres, which are normally anchored to paxillin-containing focal adhesion sites, disintegrated. Following depletion of CTEN (through siRNA) EGF-stimulated cells presented large, densely packed colonies that resembled the morphology of cells grown in the absence of EGF. This CTEN depletion also resulted in the restoration of a dense network of actin fibres, even in the presence of EGF in the growth medium. In conclusion, EGF-driven movement was shown to be dependent on the induction of CTEN and reorganization of the actin cytoskeleton. As aberrant expression and activation of EGFR are implicated in breast cancer progression, the potential role of CTEN in cancer was assessed in 272 cases of invasive breast carcinomas. CTEN expression was significantly correlated with EGFR and HER2 overexpression, and was inversely correlated with the presence of estrogen receptor. CTEN expression was also highly related with higher tumour grade and with the ability of metastasizing to the nearby lymph nodes. These data implicate CTEN in breast cancer progression and identifies it as a potential marker of a poorly differentiated, aggressive population of breast cancers, driven by EGFR/HER2 signalling. From the analysis of a small cohort of patients with HER2-positive inflammatory breast carcinomas, CTEN expression was observed in 56% of the tumour biopsies. In addition, CTEN expression was also highly related with EGFR activation. The effect on CTEN was also examined in sequential inflammatory breast cancer biopsies obtained prior and during lapatinib therapy. In all the cases a significant downregulation of CTEN was observed. The proposed model representative of the functions of CTEN and TNS3 in promoting EGF-induced cell migration is depicted in Figure 5e of Paper VIII. Recently another group analysed CTEN expression in a larger cohort of breast cancer patients [32]. These authors also found CTEN to be associated with both higher tumour grade and the ability to metastasize to axillary lymph nodes. Moreover they showed that CTEN overexpressing tumours presented a

shorter breast cancer specific survival and distant metastasis free interval, which supports the oncogenic role of CTEN in breast cancer. CTEN was also found to be upregulated in thymomas [33] and in lung cancer [34] and more recently was associated with colorectal carcinomas as it correlates with altered cell morphology and increased cell motility through repression of E-cadherin [35].

The phosphatidylinositol phosphatase SYNJ2 was also found to be upregulated in the MCF10A EGF stimulated cells as opposed to the serum stimulated ones. Further refinement on the possible role of this protein in breast cancer progression was obtained from the observation that SYNJ2 was also upregulated on the highly metastatic breast cancer cell line MDA-MB-231 LM2, which provided further evidence on its involvement in breast cancer progression. By siRNA, it was observed that the depletion of SYNJ2 was sufficient to block both migration and invasion in MCF10A cells even in the presence of EGF. Analysis of an *in vivo* mouse model demonstrated that the ablation of SYNJ2 was sufficient to block lung metastasis whereas the re-expression of the protein restored the metastatic capability. Taken the observed roles of SYNJ2 both *in vitro* and *in vivo* models the status of SYNJ2 in breast cancer samples was of interest. SYNJ2 expression was mainly observed in basal-like and HER2-overexpressing tumour groups, in agreement with an expected association with a more aggressive phenotype. SYNJ2 functions in clathrin-mediated endocytosis and has been localized in many cellular compartments like the outer mitochondrial membrane, the plasma membrane, amongst others. This may account for the different expression patterns of SYNJ2 in basal-like tumours (in the cytoplasm) and HER2 overexpressing tumours (in the membrane). When analysing the different clinicopathological parameters it was observed that SYNJ2 positive cases were more frequently ER negative and HER2 positive. Although SYNJ2 did not correlate with any other tumour features, it was possible to observe that SYNJ2 positive tumours more frequently presented distant metastasis. Taken together these results provide important insights to the role of SYNJ2 in breast cancer progression.

Finally, in Paper IX the role of the negative regulator of EGFR signalling, LST2 was assessed. In order to achieve fidelity of signalling outcomes activated RTKs, such as EGFR, are subjected to signal desensitizing mechanisms. Growth factor binding to EGFR accelerates its internalization through clathrin-coated pits which is followed by the efficient lysosomal targeting of internalized receptors and results in receptor down-regulation [36]. Yet, mechanisms that govern endocytosis are still incompletely understood. In this study a unique model of regulation, was described. Proteins that contain a FYVE domain, like hLST2, are more often targeted to PI3P-enriched membranes. However the steady-state distribution of hLST2 did not coincide with the patterns

observed in other family members. Mutational analysis of the specific ubiquitin acceptor site in hLST2 showed that it was monoubiquitylation that inhibited its binding to PI3P. The proposed mechanism is: LST2 protein is localized in PI3P-enriched early endosomes, through the binding of its FYVE domain. Upon monoubiquitylation at a conserved lysine residue (Lys⁸⁷), the FYVE domain is impaired and LST2 is sequestered from the endosomes. So, it is an ubiquitylation/deubiquitylation cycle that allows intermittent recruitment of LST2 to sorting endosomes during EGFR trafficking. Given its negative role in EGF signalling, the status of hLST2 was assessed in breast carcinomas. In a series of 272 invasive breast carcinomas, hLST2 was observed in 4.4% of the cases. Due to the low number of positive cases it was not possible to correlate LST2 expression with clinicopathological parameters. Still, positive cases were more frequent in the luminal A tumour subtype, relatively differentiated and displayed limited metastasis to adjacent lymph nodes, which was in line with the putative tumour suppressor function of hLST2. The low number of LST2 positive cases raises the possibility that in breast cancer, LST2 might not be the preferential negative regulator. Another hypothesis is that LST2 is specifically related to EGFR regulation and not with other family members (like for example HER2), and the analysed series presents few EGFR positive cases (as described in Chapter 2). Future studies are needed to address other roles of LST2, and associated protein complexes, in the sorting of RTKs to degradation or recycling.

The cellular response to stimulation involves the induction of a limited set of forward-signalling events, followed by a large cellular effort focused on signal attenuation in order to achieve tight control of the system. The better characterization of these signalling networks through their inputs, processing machinery, regulators and outputs will enable a comprehensive understanding of the system that will allow the prediction of its behaviour, enabling many of the systems constituents to be targets for therapy.

KEY FINDINGS

- In invasive breast cancer, CTEN expression correlated with EGFR and HER2 overexpression, and with lymph node metastasis. It was also more frequently observed in basal-like breast carcinomas;
- SYNJ2 was more frequently expressed in basal-like breast carcinomas and HER2-overexpressing tumours;
- LST2, a negative regulator of EGFR signalling, was expressed in 4.4% of cases mainly of the luminal A subtype.

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SUMMARY AND CONCLUSIONS

BASAL-LIKE BREAST CARCINOMAS: KNOWING THE MARKERS TO ASSESS

Breast cancer heterogeneity initially observed through tumour histology, clinical presentation and response to therapy was also captured at a molecular level when gene expression profiles revealed the so-called “molecular portraits” [1-4]. With the genetic signatures came the promise of a new era of individualized management for patients. These gene expression profiles of breast cancer have identified specific subtypes with clinical, biologic and therapeutic implications: luminal A, luminal B, HER2-overexpressing and basal-like. The latter group, basal-like carcinomas, has captured the attention of oncologists and scientists as no specific therapeutic regimen is currently available for their treatment. Both luminal and HER2-overexpressing tumours are expected to respond to either endocrine therapy or to monoclonal antibodies or tyrosine kinase inhibitors, respectively [5]. Understanding the underlying mechanisms that drive development and progression of basal-like breast carcinomas is still far. However the huge amount of information and knowledge acquired since their “re-discovery” through gene expression profiles is undeniable. In order to accelerate this process, research efforts need to be employed in unifying strategies for their identification in order to provide treatment recommendations that are truly molecularly tailored for each individual.

Aiming at a deeper insight into basal-like breast carcinomas aetiology, in the present thesis invasive breast carcinomas were classified into the different subtypes of breast carcinomas, using the classification described by Nielsen *et al* [6]. Basal-like breast carcinomas corresponded to 9.2% of the cases, were of higher grade, presented a higher proliferation rate, were more frequently associated with the development of locoregional and distant metastasis and presented worse overall survival.

As still no consensual immunohistochemical panel for the definition of basal-like breast carcinomas is accepted, other markers like P-cadherin, CK14 and Vimentin were also assessed. All these markers have been associated with basal-like phenotype either through their higher expression in the myoepithelial/basal layer (like P-cadherin or Vimentin) or through their association with worse prognosis and with the morphological features of basal-like carcinomas (like CK14). It was demonstrated that all three markers are able to recognize basal-like breast carcinomas particularly when associated with CK5. P-cadherin can also recognize possible basal-like tumours among triple negative tumours that do not express either CK5 or EGFR.

EGFR: HINTS AND PRESUMPTIONS

The predictive and prognostic significance of immunohistochemically defined basal-like breast carcinomas is still ill-defined. However, several potential targets, like EGFR, have emerged. Encouraged by the newly developed therapies against EGFR, its expression in invasive breast carcinomas was of interest. For that we studied the series of invasive breast carcinomas (to validate EGFR association with basal-like carcinomas) as well as a specific subgroup of breast carcinomas that was previously shown to present basal-like features, the metaplastic breast carcinomas. When analysing the invasive breast carcinoma series EGFR expression was only observed in 20 out of 353 cases (5.7%) but was in fact more related to basal-like tumours than to the other subtypes. In metaplastic breast carcinomas EGFR was overexpressed in 68% of the analysed cases. One possibility is that EGFR expression might be more related to specific histological types of breast carcinomas rather than being a throughout expressed marker. This may explain the not entirely congruent results of EGFR expression that appear in the literature, that range from 14-91% [7]. Next we analysed possible mechanisms that lead to EGFR overexpression in metaplastic breast carcinomas. By chromogenic *in situ* hybridization we observed that 34% of the cases presented gene amplification. As genetic amplification was not observed in the majority of the cases with receptor overexpression, we also searched for the presence of activating mutations in EGFR. None were found. The exact mechanism for receptor overexpression in 64% of the cases still remains to be uncovered. Some possible mechanisms could be ligand overexpression or defects in the sorting machinery that might not target the receptor for degradation or might enhance its recycling back to the membrane.

SIGNALLING PATHWAYS – A WAY TO SUSTAIN MALIGNANCY

Other potential hallmarks of disease, like signalling pathways activation, are also under severe scrutiny. Most of the pathway effectors represent new opportunities for therapeutic targeting. EGFR and other basal-like phenotype associated proteins are known to be activators of PI3K and MAPK pathways. Both have already established their important role in cancer progression mainly through their association with important cellular processes like, proliferation, growth, survival, metabolism among others [8]. In order to determine the importance of these signalling pathways in the development and progression of basal-like breast carcinomas some of their effectors were assessed. From PI3K pathway, p110 α , p110 β , p-AKT, p-mTOR, p-NF κ B as well as the PI3K antagonist PTEN, were analysed. p110 α was more frequently expressed in basal-like breast carcinomas. However when considering invasive breast carcinomas as a whole, p110 α 's

expression was more frequently observed in hormone receptor positive cases. Very few studies have addressed p110 α 's expression preferring the assessment of the mutational status of its codifying gene *PIK3CA*. In breast, *PIK3CA* mutations are frequent but their correlation with prognosis is still debatable. Depending on the study they can correlate with both good and bad prognostic markers. When investigating the invasive breast carcinoma series, a 19.2% mutation frequency was encountered. *PIK3CA* mutations were more frequent in exon 20, and were present in both luminal A and basal-like breast carcinoma cases. Surprisingly there was not an association between presence of mutation and protein expression (p110 α). This discrepancy in results and the huge amount of contradictory results and associations only confirms the need to validate in large cohorts of patients, the actual biological significance of *PIK3CA* mutations or p110 α 's expression. The other PI3K catalytic subunit assessed, p110 β , was not a major player in basal-like breast carcinomas. However its striking association with HER2 positivity makes this protein a good candidate for future studies in this setting. It would also be of interest to determine the precise role of p110 β in cells, in order to define the importance of the staining patterns observed (membrane and granular). Probably these can relate to the described kinase dependent or kinase independent function attributed to p110 β [9].

AKT has been shown to be the primary downstream mediator of the effects of the PI3K processes and through the phosphorylation of target substrates AKT impinges numerous cellular processes [10]. Activation of AKT was observed in the majority of the analysed cases, irrespectively of their molecular subtype. However AKT activation was not dependent on the expression of PI3K catalytic subunits. This can be explained by AKT activation through signalling modules, like RAS and SRC [11]. As for AKT downstream partners p-mTOR and p-NF κ B neither seemed to be of paramount importance for basal-like carcinomas sustained development or progression. The majority of basal-like breast carcinomas were negative for both proteins.

Signalling pathway activation can also occur due to the lack of their negative regulators, which in the case of PI3K pathway is the tumour suppressor PTEN. PTEN has for long been associated with breast cancer either due to the high mutation rate encountered or to the high percentage of promoter methylation. PTEN is also an important predictive marker of response to therapy. More recently PTEN has been associated with basal-like carcinomas. For all the above reasons the assessment of PTEN expression in invasive breast carcinomas was of importance. PTEN expression was evaluated based on 4 observed phenotypes: negative, reduced, normal and nuclear. All the comparisons were made using PTEN expression in blood vessels as control. PTEN was negative in 36.6% of the cases. However it was in basal-like carcinomas that this

absence of expression was more pronounced as 64.5% of the cases were negative. From all the studied alterations in this pathway PTEN lack of expression was without a doubt the most pronounced alteration found in basal-like breast carcinomas.

When analysing MAPK activation, through the expression of its downstream effectors, p-ERK1/ERK2, it was possible to observe that this is not a preferential pathway in basal-like carcinomas. Only 19.2% of the cases presented activated p-ERKs. Interestingly, although not statistically significant the strong expression of p-ERKs in the stroma (particularly in HER2-overexpressing tumours) reinforces the idea of the stromal involvement in the acquirement of a more aggressive phenotype, representative of possible epithelial-stromal interactions.

BUILDING A WEB OF KNOWLEDGE

RTK modules are frequently mutated or amplified in cancers for which its interception can retard or block tumour growth. However from clinical experience, targeting RTK signalling also brings with it the evolvement of drug resistance that can be seen as a manifestation of plasticity of robust networks [12]. This systems level of understanding of signalling networks can enable the identification of possible therapeutic targets.

By using a systems biology approach to address dynamics of growth factor signalling, Amit *et al*, provided a tool to further identify EGFR pathway components. From his data set two molecules, CTEN and SYNJ2, appeared as being upregulated through the EGFR system and both could be potentially involved in migration and metastasis of breast cancer. The analysis of both proteins allowed further insight into the acquirement of a more aggressive phenotype. When analysed in invasive breast carcinomas, CTEN expression was associated with the expression of EGFR and HER2 and was inversely correlated with ER. Furthermore, CTEN expressing tumours presented higher histological grade and frequently presented lymph nodes metastasis. CTEN positivity was mostly observed in basal-like carcinomas. SYNJ2 was also more frequently observed in basal-like and HER2-overexpressing tumours, which is in agreement with its attributed role in a more aggressive phenotype. ER negative tumours were more frequently SYNJ2 positive and although not statistically significant SYNJ2 positive tumours presented more frequently distant metastasis than the negative ones. However in order to ensure fidelity of the signalling outcomes these networks are also subjected to signal desensitizing mechanisms, that can entail, among others, accelerated endocytosis and degradation in lysosomes. The expression in invasive breast carcinomas of LST2, a negative regulator of the EGFR signalling was also determined. LST2 was expressed in 4.4% of the cases. It was more frequently observed in luminal A cases. LST2

positive cases were also more differentiated and displayed limited metastasis to adjacent lymph nodes, in line with its putative tumour suppressor activity.

The integrated signalling networks and phenotypes arising from EGFR activation are still poorly annotated. However, these systems-based approaches will be useful in identifying players with positive impact in the development of network-based therapeutics with translation into the clinic.

BASAL-LIKE BREAST CARCINOMAS: WHERE TO GO NOW?

Other RTKs, like c-KIT have also been associated with basal-like carcinomas [6, 13, 14]. c-KIT has also been successfully targeted in other tumour types by imatinib that inhibits c-KIT as well platelet-derived growth factor receptor tyrosine kinases [15]. Yet these findings should be looked with caution as responses to c-KIT's therapy are attributed to the presence of activating mutations in this receptor, which are rare in breast cancer [14]. However when looking at the literature about basal-like carcinomas the most discussed molecule linked with this subgroup of tumours is *BRCA1*. Many phenotypical, immunohistochemical, clinical, as well as molecular features are shared between basal-like tumours and tumours that arise in carriers of *BRCA1*-germline mutations. The majority of *BRCA1* mutated tumours are triple negative and express basal CKs, in addition to other commonly expressed basal-like markers like p53, P-cadherin and EGFR [16-23]. Expression profiles also cluster *BRCA1* tumours in the basal-like subgroup [16]. Although *BRCA1* mutations are rare in sporadic tumours [24], it is observed that in these cases epigenetic mechanisms like, *BRCA1* promoter methylation [25, 26] or a dysfunction in the upstream pathway that regulates its expression, like overexpression of ID4 [27] can impair its function. These sporadic tumours with lower *BRCA1* expression tend to present the same features as those observed for *BRCA1*-mutated and basal-like tumours [23, 27-29]. There is increasing evidence that DNA-repair defects characteristic of *BRCA1*-related cancer, especially those related to defective homologous recombination confer sensitivity to certain systemic agents, such as platinum salts based chemotherapy and Poly-ADPribose polymerase (PARP) inhibitors [30-33], which could be used for the treatment of breast cancer. There are several ongoing clinical trials to assess their efficacy in this tumour population.

All these observations point to the same conclusion: cancer is a complex disease, and if a lesson needs to be taken from the huge amount of available data provided by the genome-wide analysis is that the time to look for individual genes or proteins is over. This is probably the reason why cancer cure has had limited success. Why do you get a result in a cell line but are rarely able to reproduce it in a patient? Why after an initial response sometimes you get relapses? Why after

inhibiting one signalling pathway another one is activated? It is an integrated and interacting network of genes that will cause a specific phenotype. The use of this knowledge will allow “getting closer to the whole picture” [34].

KEY FINDINGS

- Establishment of an invasive breast carcinoma tumour bank classified according to the different subtypes of breast carcinomas: luminal A, luminal B, HER2-overexpressing and basal-like;
- Basal-like breast carcinomas were grade III, had high proliferation rates and frequently presented both locoregional and distant metastasis;
- P-cadherin, CK14 and Vimentin proved to be useful biomarkers in the identification of basal-like breast carcinomas;
- P-cadherin was able to rescue possible basal-like breast carcinomas among triple-negative cases that do not express either CK5 or EGFR;
- EGFR expression was more frequently observed in basal-like breast carcinomas than in other subtypes;
- EGFR was overexpressed in 68% of metaplastic breast carcinomas and in 34% of these gene amplification was the underlying mechanism;
- Lack of PTEN expression was a frequent event in basal-like breast carcinomas, being observed in 64.5% of the cases;
- p110 β appeared as a new target in breast cancer, particularly in HER2-positive cases, as it was expressed in 93.8% of this cases;
- AKT was activated in the majority of breast carcinomas, accounting for its integrating role in cells;
- pmTOR and pNF κ B were not major players in basal-like breast carcinomas as most did not express these proteins;
- MAPK activation was mainly observed in luminal A tumours (52.5%) as opposed to basal-like breast carcinomas (19.4%);
- In invasive breast cancer, CTEN expression correlated with EGFR and HER2 overexpression, and with lymph node metastasis. It was also more frequently observed in basal-like breast carcinomas;
- SYNJ2 was more frequently expressed in basal-like breast carcinomas and HER2-overexpressing tumours;
- LST2, a negative regulator of EGFR signalling, was expressed in 4.4% of cases mainly of the luminal A subtype.

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