Gene Expression Analysis in Human Breast Cancer

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Breast Cancer is the most commonly diagnosed cancer in women, with more than 1.2 million women diagnosed annually worldwide. It is also a frequently fatal disease and remains difficult to treat, despite advances in all facets of cancer management. While a number of genetic mutations have been identified in human breast cancers, the specific combinations of the mutations required in concert for formation of a breast carcinoma remains unknown, making precise detection or prognostic predictions impossible. Although estrogen receptor (ER) status is predictive of response to hormonal treatments, there are currently no clinically useful predictive markers of a patient’s response to chemotherapy. This results in all patients who are eligible for chemotherapy receiving the same treatment even though de novo drug resistance will result in the treatment failing in about 80% of cases. Developing improved diagnostic tools to cluster different breast cancers into groups based on genetic parameters has the potential to revolutionise individualised treatment options and subsequent efficacy. This in turn will improve quality of life for patients undergoing therapy who will no longer suffer the consequences of unnecessary treatments and more importantly, a subsequent improved survival rate.

Genetic research strategies related to breast cancer have involved attempts at distinguishing genes involved in susceptibility such as BRCA-1, BRCA-2, HER-2 and the nuclear receptor gene superfamily. A powerful approach in molecular genetics is the determination of gene expression which can provide investigators with a profile of gene expression in healthy tissues which is then compared to disease tissues to identify key determinants of disease progression. Gene expression profiling has demonstrated the heterogeneity of breast cancers, that large variability in biology in turn throwing the difficulty of developing more effective treatments into sharp relief. Technologies such as microarrays and real time quantitative polymerase chain reaction (Q-PCR) have been used to detect specific genes that may act differently in breast cancer patients compared to healthy controls. The enormous improvements in scale that these technologies has provided has opened the door to potential personalisation of treatment, despite cancer heterogeneity.
The goal of improving systemic treatment of breast cancers is to evolve from a generic approach, treating every patient with a ubiquitous, relatively non-specific cytotoxic chemotherapy or hormonal therapy, to a more individually-tailored direct treatment. One method for improving personalisation of therapy is to examine molecular events that define specific sub-types of breast cancer with potentially unique prognostic implications. Although anatomic staging and histological grade are important prognostic factors, they often fail to predict the clinical course of this disease. In order to improve upon the standard of care for breast cancer, there is a need for new molecular markers and diagnostic algorithms. This study was aimed at defining gene expression changes associated with breast cancers of differing grades using microarrays and undertaking further Q-PCR, immunohistochemistry and genotyping studies of implicated genes. mRNA was extracted from formalin fixed paraffin embedded archival breast invasive ductal carcinoma tissue samples of progressive grades (Grades I-III), including benign tumours of three samples per grade and three benign tissues for the microarray analysis. Gene expression profiles were determined by microarray using the Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays and validated by Q-PCR using a Corbett RotorGene 6000 in the same tumour population as well as in an extended tumour population. Following validation, the gene expression profile of the identified targets was also investigated in the human breast cancer cell lines MCF-7 and MDA-MD-231. Immunohistochemistry (IHC) was also used to detect the AXIN2 protein in all tissues. Single nucleotide polymorphisms within some of the more significant target genes derived from the microarray studies as well as some from other studies were genotyped using High Resolution Melt analysis within a breast cancer affected and healthy matched controls population.

In the first part of this thesis, we undertook gene expression investigations in grade III tumours compared to benign tumours using microarray technologies and validation using Q-PCR. From the array data, an independent group t-test revealed that 502 genes were found to be significantly (p≤0.05) differentially expressed between grade III tumour and control tissues (see Appendix B). Seven of these genes were found to be involved in the Wnt signalling pathway which is known to participate in cell fate specification and differentiation. Down-regulated genes included AXIN2, TCF7L2 and LDLR, while DUSP13, MARK4, PPP3CA and CSNK1E were significantly up-regulated in the grade III tumours when compared to the benign tumours. Three of the genes (MARK4, PPP3CA and LDLR) that showed gene expression changes in the microarray data were found to be replicated in subsequent Q-PCR analysis using the same samples, as well as when additional samples were added to the
analyses. *In vitro*, PPP3CA continued this trend of gene expression in the tested breast cancer cell lines.

In the second gene expression study we undertook investigations in tumours of all grades (I-III) including benign tumours using microarray analysis and validation using Q-PCR and immunohistochemistry. Independent group t-tests revealed that 178 genes were significantly (p≤0.01) differentially expressed between the three grades of malignant breast tumours when compared to benign tissues. Six tumour group comparisons were made between the three grades and benign tumours. From these results, eight genes were shown to be significantly differentially expressed in more than one comparison group. All eight of these genes have been shown to be involved in processes implicated in breast cancer development and/or progression. Genes up-regulated in grade I tumours were CLD10 and ESPTI1, with the expression level statistically significant in both microarray and the validation Q-PCR analyses. The expression of the CDC42EP3, ZAN, TCEA3 and PALMD genes were significantly up-regulated in grade III tumours, with this trend in expression replicated by Q-PCR analysis, although not significantly. CXCL16 gene expression was up-regulated in benign tumours while the AXIN2 gene was differentially expressed in all grades and benign tumours in the microarray analysis, however this trend was not replicated via Q-PCR. The IHC analysis of AXIN2 showed a general gradual decrease of intensity with increasing tumour grade.

The final gene expression study we undertook investigated gene expression differences between grade III compared to benign tumours as well as grade I tumours compared to grade II tumours using microarray analysis and validation using Q-PCR. The array data revealed that 888 genes were found to be significantly (p≤0.05) differentially expressed between grade I and II tumours and 502 genes between grade III and benign tumours (see Appendix B). As part of this research, the role of four semaphorin/plexin signalling genes from the microarray analysis in human breast cancers *in vivo* and *in vitro* were investigated. The microarray analysis revealed that SEMA4D was significantly (P=0.0347) down-regulated in the grade III tumours compared to benign tumours; SEMA4F, was significantly (P=0.0159) down-regulated between grade I and II tumours; PLXNA2 was significantly (P=0.036) down-regulated between grade III and benign tumours and PLXNA3 significantly (P=0.042) up-regulated between grade I and II tumours. Gene expression of SEMA4D was validated using Q-PCR, demonstrating the same expression profile in both data sets. When the sample set was increased to incorporate more cases, SEMA4D continued to follow the same expression profile, including statistical significance for the differences observed and small standard
deviations. *In vitro* the same pattern was present, where expression for SEMA4D was significantly higher in MDA-MB-231 cells when compared to MCF-7 cells.

Following these expression studies, a pair of association studies were undertaken, in an association population consisting of Caucasian women affected by breast cancer and a population of controls, matched for age, sex and ethnicity. These studies investigated SNPs in several genes which had previously been implicated in breast cancer progression in our tissue population above.

The first genotyping study we undertook was concerned with two single nucleotide polymorphisms (SNP) in the NCOA3 gene which has widely been associated with breast cancer. NCOA3 is a known low to moderate-risk breast cancer susceptibility gene, amplified in 5-10% and over expressed in about 60% of breast tumours. Additionally, this over expression is associated with Tamoxifen resistance and poor prognosis. Two variants of NCOA3, 1758G>C (rs2230782) and 2880A>G (rs2076546) have been previously associated with breast cancer in two independent populations. Here we assessed the influence of the two NCOA3 variants on breast cancer risk by genotyping an Australian case-control study population. 172 cases and 178 controls were successfully genotyped for the 1758G>C variant and 186 cases and 182 controls were successfully genotyped for the 2880A>G variant using high-resolution melt analysis (HRM). The genotypes of the 1758G>C variant were validated by sequencing. Chi-square tests were performed to determine if significant differences exist in the genotype and allele frequencies between the cases and controls. Chi-square analysis returned no statistically significant difference (p >0.05) for genotype frequencies between cases and controls for 1758G>C ($X^2=0.97$, p=0.6158) or 2880A>G ($X^2=2.09$, p=0.3516). Similarly, no statistical difference was observed for allele frequencies for 1758G>C ($X^2=0.07$, p=0.7867) or 2880A>G ($X^2=0.04$, p=0.8365). Haplotype analysis of the two SNPs also showed no difference between the cases and the controls (p=0.9585).

The final genotyping study we undertook was concerned with two SNPs from the candidate genes which emerged from the Wnt signalling pathway investigation. Previously, we identified two Wnt signalling pathway-implicated genes, PPP3CA and MARK4, as having a role in more aggressive and potentially metastatic breast tumours. In this study, we examined two SNPs within PPP3CA and MARK4 in an Australian case-control study population for a potential role in human breast cancers. 182 cases and 180 controls were successfully genotyped for the PPP3CA SNP (rs2850328) and 182 cases and 177 controls were successfully genotyped for the MARK4 SNP (rs2395) using High Resolution Melt (HRM)
analysis. Genotypes of randomly selected samples for both SNPs were validated by dye terminator sequencing. Chi-square analysis showed no statistically significant difference (p>0.05) for genotype frequencies between cases and controls for rs2850328 ($X^2=1.2$, p=0.5476) or rs2395 ($X^2=0.3$, p=0.8608). Similarly, no statistical difference was observed for allele frequencies for rs2850328 ($X^2=0.68$, p=0.4108) or rs2395 ($X^2=0.02$, p=0.893).

The two most implicated candidates derived from the Wnt investigation were MARK4, PPP3CA and LDLR as they were able to be replicated in Q-PCR analysis of the original tumour samples and continued to have the same significant mode of expression in the extended population. AXIN2 and TCF7L2 and their association with breast cancer have already been widely documented in the literature, an observation supported in our Q-PCR analyses. The expression of PPP3CA in MDA-MB-231 cells was significantly higher (p=0.0017) compared to expression in the less invasive MCF-7 cell line, following the same trend in gene expression as the in vivo data. The consistency of PPP3CA in both in vivo and in vitro data from this study, make it a likely candidate for further investigations. While our target genes require further validation, the ability to define genetic differences of breast cancers using microarray technology may enable us to define more clearly markers of susceptibility, development and/or progression and may have important clinical implications in terms of early diagnosis and treatment.

From the study concerning genes expressed in multiple grade comparisons, the two most implicated genes as candidates for a role in differentiating less aggressive and potentially metastatic breast tumours, were CLD10 and ESPTI1. Their gene expression profile from the microarray analysis was not only replicated in Q-PCR analyses of the original tumour samples, but continued to demonstrate a significant trend of gene expression in an extended sample population. AXIN2 was not only involved in all cancer grades on a genomic level but on a proteomic level as well. The IHC for AXIN2 revealed no significant variation in signal intensity between the grades. However, a significant association between AXIN2 protein expression and ER status was revealed. While our microarray and Q-PCR analyses linked CXCL16, CDC42EP3 and ZAN with either grade III tumours (CDC42EP2), benign tumours (CXCL16) or both (ZAN), these changes were not significant when validated by Q-PCR. Although significant by microarray analysis, TCEA3 and PALMD validation by Q-PCR was also inconsistent.

From our Semaphorin/plexin investigation, the expression of SEMA4F, PLXNA2 and PLXNA3 could not be validated using Q-PCR, however in vitro analysis of these three genes
revealed that both SEMA4F and PLXNA3 followed the microarray trend in expression, although they did not reach significance. In contrast, PLXNA2 demonstrated statistical significance in the in vitro experiment and was in concordance with the literature. We, and others, have proposed SEMA4D to be a gene with a potentially protective effect in benign tumours that contributes toward tumour growth but also metastatic suppression. Data here supports a role for SEMA4F as a tumour suppressor in the peripheral nervous system but is implicated in tumour progression in breast cancer. Our in vitro analysis of PLXNA2 indicated that the gene indeed does promote proliferation in breast cancer. Finally, our in vitro analysis on PLXNA3 also suggest that this gene may also act as a tumour suppressor in breast cancer, in addition to a similar role for the gene previously reported in ovarian cancer. In this study, SEMA4D is implicated to have a role in more aggressive and potentially metastatic breast tumours. Semaphorins and their receptors, the plexins, have been implicated in numerous aspects of neural development, however their expression in many other epithelial tissues suggests that the semaphorin-plexin signalling system also contributes to blood vessel growth and development. These findings warrant further investigation of the role of semaphorins and plexins and their role in normal and tumour-induced angiogenesis in vivo and in vitro. This may represent a new front of attack in anti-angiogenic therapies of breast and other cancers.

Our findings for the NCOA3 genotyping study in an Australian Caucasian population composed of breast cancer sufferers and an age matched control population did not support the findings of previous studies demonstrating that these markers play a significant role in breast cancer susceptibility. Here, no significant difference was detected between breast cancer patients and healthy matched controls by either the genotype or allele frequencies for the investigated variants (all p≥0.05). While an association of the two variants and breast cancer was not detected in our case-control study population, exploring these variants in a larger population of the same kind may obtain results in concordance with previous studies. Given the importance of NCOA3 and its involvement in biological processes involved in breast cancer and the possible implications variants of the gene could have on the response to Tamoxifen therapy, NCOA3 remains a candidate for further investigation.

The analysis from the Wnt SNP genotyping study did not find a positive association between these markers and breast cancer susceptibility in our population as no significant difference could be detected between the cases and healthy matched controls by either the genotype or allele frequencies for the investigated SNPs (all p≥0.05). Even though an association of the polymorphisms rs2850328 and rs2395 and breast cancer was not detected in our case-control study population, other variants within the PPP3CA and MARK4 genes may still be
associated with breast cancer, as both genes are implicated with processes involved in the disease as well as their mutual partaking in the Wnt signalling pathway.

It is readily acknowledged and established that significant differences exist in gene expression between different cancer grades. Expansion of the approaches to gene expression and association studies detailed herein, especially using narrowed pathway specific approaches may lead to an improved ability to discriminate between cancer grade and other pathological factors. In turn, defining genetic differences in breast cancer may enable us to more clearly find markers of susceptibility, development and/or progression and in turn improve clinical implications in terms of early diagnosis and treatment.
STATEMENT OF ORIGINALITY

The material presented in this report has not previously been submitted for a degree diploma in any University, and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgement is made in the Thesis itself.

This thesis includes published first author papers as individual chapters, which is an accepted format of thesis submission and involves work undertaken by myself with the exception of microarray hybridisation undertaken at Australian Genome Research Facility and primary analysis of microarray data undertaken by Dr Thomas Tiang at the Genomic Research Centre.

_______________________
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I would like to dedicate this work to breast cancer sufferers and their families.
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List of Abbreviations

18s – 18s Ribosomal RNA Gene, commonly used reference gene
AGRF – Australian Genome Research Facility
AIB1 - amplified in breast 1, alternative name for NCOA3
AKT - serine/threonine protein kinase
ANOVA – analysis of variance
APC - Adenomatosis Polyposis Coli Gene
ATCC – American Type Cell Culture
AXIN2 – Axin 2 Gene
Bp – base pair
CDC42EP3 - CDC42 effector protein 3
C/EBPβ - family of transcription factors that promote the expression of certain genes through interaction with their promoter
CI – confidence interval
CLD10 - Claudin 10
CSNK1E - Casein kinase 1, Epsilon Gene
CXCL16 - Chemokine ligand 16
DNA – deoxyribonucleic acid
dNTPs - deoxynucleotide triphosphate
DUSP13 - Dual Specificity Phosphatase 13 Gene
E2F1 - protein that in humans is encoded by the E2F1 gene
EGFR - Epidermal growth factor receptor
EPSTI1 - Epithelial stromal interaction 1
ER – estrogen receptor
FFPE – formalin-fixed paraffin-embedded
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase Gene
GSK-3 - Glycogen Synthase Kinase 3
H&E – haematoxylin and eosin
HBC – Human Breast Cancer
HRM – high resolution melt
IDC – invasive ductal carcinoma
IGF-1 – insulin-like growth factor 1
LDLR - Low Density Lipoprotein Receptor (familial hypercholesterolemia) Gene
LRP - Low Density Lipoprotein Receptor Related Protein Gene
MAF – Minor allele frequency
MAPK - Mitogen-activated protein kinases
MARK4 – MAP/microtubule Affinity-Regulating Kinase 4 Gene
MMP-1 - matrix metalloproteinase-1
mRNA – messenger ribonucleic acid
NCBI - National Centre for Biotechnology Information
NCOA3 - Nuclear Receptor Co-activator 3 gene
NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells
OR – odds ratio
PALMD - Palmdelphin
PEA3 – ETS-domain transcription factor
PLXNA2 – Plexin A2
PLXNA3 – Plexin A3
PPP3CA - Protein Phosphatase 3, Catalytic subunit, Alpha isoform (calcineurin A alpha) Gene
PR – progesterone receptor
Q-PCR - quantitative real time polymerase chain reaction
RNA – ribonucleic acid
RNAi - RNA interference
RPL13A – Ribosomal Protein Ligand 13A Gene
SEMA4D – Semaphorin 4D
SEMA4F – Semaphorin 4F
SNP – short nucleotide polymorphism
SRC-3 – steroid receptor coactivator-3, alternative name for NCOA3
SRW – Stephen R. Weinstein
STAT – Signal Transducer and Activator of Transcription, family of transcription factors
TCEA3 - Transcription elongation factor 3
TCF7L2 - Transcription factor 7-like 2 (T-cell specific, HMG-box) Gene
TDLUs - terminal duct lobular units
TP53 – tumour protein 53
TRAM-1 - thyroid hormone receptor activator molecule 1, alternative name for NCOA3
WNT – wingless
ZAN – Zonadhesin
Publications Arising from Work Described in this Thesis


Gabrovska, P. N., Smith, R. A., Tiang, T., Weinstein, S. R., Haupt, L. M., & Griffiths, L. R. Genes involved in Wnt signalling are associated with more aggressive human breast tumours. [Manuscript under review in “Microenvironment Targets in Cancer Progression and Metastasis”]
Related Publications


