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Development of an eight gene expression profile implicating human breast tumours of all grade

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Abstract The goal of improving systemic treatment of breast cancers is to evolve from treating every patient with non-specific cytotoxic chemotherapy/hormonal therapy, to a more individually-tailored direct treatment. Although anatomic staging and histological grade are important prognostic factors, they often fail to predict the clinical course of this disease. This study aimed to develop a gene expression profile associated with breast cancers of differing grades. We extracted mRNA from FFPE archival breast IDC tissue samples (Grades I–III), including benign tumours. Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays were used to determine gene expression profiles and validated by Q-PCR. IHC was used to detect the AXIN2 protein in all tissues. From the array data, an independent group *t*-test revealed that 178 genes were significantly ($P \leq 0.01$) differentially expressed between three grades of malignant breast tumours when compared to benign tissues. From these results, eight genes were significantly differentially expressed in more than one comparison group and are involved in processes implicated in breast cancer development and/or progression. The two most implicated candidates genes were CLD10 and ESPTI1 as their gene expression profile from the microarray analysis was replicated in Q-PCR analyses of the original tumour samples as well as in an extended population. The IHC revealed a significant association between AXIN2 protein expression and ER status. It is readily acknowledged and established that significant differences exist in gene expression between different cancer grades. Expansion of this approach may lead to an improved ability to discriminate between cancer grade and other pathological factors.

Keywords Microarray · Q-PCR · Axin 2 (AXIN2) · CLD10 (Claudin 10) · EPSTI1 (Epithelial stromal interaction 1)

Abbreviations

AGRF	Australian Genome Research Facility
ANOVA	Analysis of variance
APC	Adenomatosis Polyposis Coli Gene
AXIN2	Axin 2 Gene
Bp	Base pair
CDC42EP3	*CDC42 effector protein 3*
CLD10	*Claudin 10*
CXCL16	*Chemokine ligand 16*
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EPSTI1	*Epithelial stromal interaction 1*
ER	Estrogen receptor
FFPE	Formalin-fixed paraffin-embedded
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase gene
H&E	Haematoxylin and eosin
IDC	Invasive ductal carcinoma
mRNA	Messenger ribonucleic acid
PALMD	*Palmdelphin*

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Q-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RPL13A	Ribosomal Protein Ligand 13A Gene
SRW	Stephen R. Weinstein
TCEA3	*Transcription elongation factor 3*
ZAN	*Zonadhesin*

Introduction

Breast cancer is a heterogeneous disease arising from multiple genetic changes in genes with pivotal roles in the homeostatic control of mammary epithelial cell proliferation, differentiation and death [1]. One of the most common malignancies worldwide, breast cancer is a leading cause of death among women, with the sporadic form of the disease constituting more than 90% of all breast cancers. Breast tumourigenesis consists of genetic changes resulting in altered mRNA and protein levels, such as the activation or amplification of oncogenes or the loss of tumour suppressor genes [2]. The level of expression of every gene involved during the malignant transformation of a cell is controlled by the transfer of information encoded in its genetic blueprint to its environment via signal transduction and regulatory processes [2].

Current management of breast cancer is based on anatomic staging which includes characteristics such as tumour size, node involvement and metastasis as well as morphological features including tumour grade [3]. In contrast to tumour stage, different tumour grades have been associated with distinct gene expression signatures [4]. Tumour grade has been a highly valuable prognostic indicator for breast cancer diagnoses, as poorly differentiated, high grade DCIS or IDC lesions are associated with significantly poorer clinical outcome [4]. Indeed, previous studies have demonstrated a subset of genes associated with high tumour grade to be quantitatively correlated with the transition from pre-invasive to invasive tumour growth with different tumour grades associated with distinct transcriptional signatures linking tumour grade with the DCIS-IDC stage transition [4]. Further investigation of the signalling pathways that link these genes to the processes of tumour grade and stage progression may provide key insights into the molecular mechanism driving breast tumourigenesis. While histological grade in breast cancer provides important prognostic information, its variability and poor reproducibility, especially for grade II tumours, have limited its clinical potential [5]. Although anatomic staging and histological grade are important prognostic factors, they often fail to predict the clinical course of the disease. In order to improve upon the standard of care for breast cancer sufferers, there is a need for new molecular markers and diagnostic algorithms [3].

The most integrated approach towards understanding multiple molecular events and mechanisms by which cancer may develop, is the application of gene expression profiling using microarray technologies [6]. As the process of oncogenesis involves the disruption of diverse cellular pathways including cell cycle, growth, survival and apoptosis, the high throughput of microarray analyses provides a powerful tool with which to examine multiple cellular processes simultaneously [7]. The differentially regulated synthesis and degradation of RNA molecules forms hierarchal systems which determine organ, tissue and cell function through complex and interactive pathways. Therefore molecular abnormalities that control gene transcription may contribute to tumour phenotype [8]. Since the development of microarray technologies, together with the advancement of RNA extraction and amplification methods, gene expression studies have revolutionised the means of discovering genes suitable to target for drug development and individualised breast cancer treatment. As of the mid-1990s, expression microarrays have been extensively applied to the study of cancer and no cancer type has seen as much genomic attention as breast cancer [9]. The most abundant area of breast cancer genomics has been the clarification and interpretation of gene expression patterns that underlie biological and clinical properties of tumours. For this reason, traditional techniques which focus on a single gene or a limited group of genes must be used within the context of larger more comprehensive methodologies in order to detect complex gene interactions. In addition, the identity of genes whose expression has been altered should then be validated through comparison of gene content in test and control tissues [10], along with validation using targeted methodologies.

Here, we examined archival formalin-fixed, paraffin-embedded (FFPE) breast cancer samples of differing grades (grades I–III) in comparison to control benign breast tissue using a whole genome profiling approach, to identify differentially expressed genes. Following extraction of RNA from tissues, samples were hybridised onto Affymetrix GeneChip® Human Genome U133 Plus 3.0 arrays to determine their gene expression profiles. Eight genes associated with breast cancer appeared highly significant ($P \leq 0.01$) across more than one grade comparison in the microarray analysis and were validated in the same population as well as an extended sample population by Q-PCR. We also analysed the protein expression of one of these genes which appeared in all grades and the benign control tissues by immunohistochemistry (IHC). From this study, we aim to develop a gene expression profile that could be used to provide insights into the complex interactions of genes involved in cancer development which in terms of

early diagnosis and treatment may have important clinical implications.

Materials and methods

Tissue population

The study population was derived from the Gold Coast region in collaboration with the Gold Coast Hospital Department of Pathology. For the microarray analysis, the population was comprised of nine archival invasive ductal carcinoma (IDC) breast tissue sections and three benign dysplasia control tissue sections embedded in paraffin and fixed with 10% buffered formalin on slides. Ten micron sections were used and compared to a haematoxylin and eosin (H&E) stained slide as a reference for tumour location in each sample. Specifically, the IDC samples were composed of three tumour samples from each of clinically diagnosed grade I, II and III and compared to three samples of benign breast tissue (dysplasia) taken from unaffected patients as the control population.

The average age of the individuals from whom the biopsies were obtained were 56.88, 59.18, 60.45 and 55.93 years for the control and grades I, II and III respectively with tumour maximum dimension ranging from 15 to 20 mm. The archival breast tissue samples were obtained through collaboration with Gold Coast Hospital Pathology with relevant ethical approvals (Approval numbers HSC/04/03/HREC and 9702 for Griffith University and Gold Coast Hospital, respectively). For consistency, cancer grading for each sample was confirmed by a single pathologist from the Gold Coast Hospital Pathology Department (SRW). There was a variety of pathological data available for the population, including immunohistochemical staining to detect ERα proteins. ERα status was determined by standard clinical criteria for treatment. The study population information is summarised in Table 1.

Table 1 Study population information

Characteristic	Microarray/original Q-PCR	Final Q-PCR	IHC
Number of tumours	12	19	20
Tumour size average (mm)	15.51	16.21	16.4
Mean age	57.31	60.89	59.4
Histological grading			
Grade I	3	4	5
Grade II	3	6	7
Grade III	3	5	6
Benign tumours	3	4	2
Estrogen-receptor (ER) status			
Positive (+)	10	13	14
Negative (−)	2	6	6
Lymph-node status			
Positive (+)	2	3	3
Negative (−)	7	12	15
In situ carcinoma			
Positive (+)	6	9	12
Negative (−)	3	6	6

RNA preparation

RNA was extracted from the tumours via an RNA Extraction Protocol devised by the Genomics Research Centre as described previously [11]. Briefly, tumour tissue was first separated from the surrounding tissue by microdissection using H&E slides immediately preceding the extracted slide as a guide to tumour location. All non-malignant tissue was removed as a single unit for separate analyses. Paraffin was removed with xylene, the tissue homogenised by passage through an 18G needle, with the sample then resuspended in TRIzol (Invitrogen). RNA was extracted according to the manufacturer's instructions. Briefly, RNA was separated from DNA and protein through the use of chloroform and centrifugation. Extracted RNA was then treated with RNasin and DNase I prior to final purification using a Qiagen RNeasy Mini Kit. cDNA was generated in a 25 µl reaction volume using 2 ng of total RNA, Superscript III (0.2 µl/reaction), random hexamers (9 µg/reaction), 5 mM dNTPs (0.5 µl/reaction) and 1× Superscript buffer (Invitrogen). The cell culture cDNA was stored until further analysis.

For microarray analyses, following the manufacturer's extraction protocol, the RNA was purified and amplified twice using the SenseAmp RNA Amplification Kit. The senseRNA was then labelled with a biotin-labelled antibody using the IVT Labelling Kit (Affymetrix), followed by ds-cDNA clean up using Sample Cleanup Module (GeneChip) and fragmented RNA using fragmentation buffer (QIAGEN). The remaining RNA was converted to cDNA for validation by Q-PCR. RNA quantity and quality was measured using an Agilent 2100 Bioanalyser (Agilent Technologies).

Microarray analysis

Microarray hybridisation was performed as previously described (Gabrovska et al. 2011) where biotin labelled and fragmented RNA was hybridised at the Australian Genome Research Facility (AGRF) onto Affymetrix HG-U133 Plus 2.0 GeneChips. Analysis methods have already been outlined in Gabrovska et al. [12]. Briefly, microarray results were analysed by a series of independent *t*-tests and one-way ANOVA analyses to determine any significant

differences in levels of gene expression in the qualitative groups. Independent *t*-test analysis was performed using the statistical tool LIMMA (Linear Models for Microarray Data) which is a software package for the analysis of gene expression microarray data, especially the use of linear models for analysing designed experiments and the assessment of differential expression [13]. The package includes pre-processing capabilities for two-colour spotted arrays. The differential expression methods apply to all array platforms and treat Affymetrix, single channel and two channel experiments in a unified way [13]. Q-PCR results were examined for significance using an independent *t*-test. The excel file with the normalised data for this research is attached as supplementary material.

Q-PCR analysis

The microarray analysis implicated eight genes associated with breast cancer ($P \leq 0.01$) across more than one grade comparison. The reference genes *GAPDH and RPL13A* were selected as assay endogenous controls for data normalisation using the GeneNorm software. Primers for the reference genes and the target genes were designed using Primer Express 3.0 (Applied Biosystems) with melting temperatures ranging from 58 to 61°C and amplicon lengths between 80 and 81 bp. Sequences for all primers designed and used in this study are outlined in Table 2.

Table 2 Genes of interest and reference gene primers for Q-PCR

Primer	Sequence (5′–3′)
AXIN2-F	TGTCTTAAAGGTCTTGAGGGTTGAC
AXIN2-R	CAACAGATCATCCCATCCAACA
CLDN10-F	TTTGCGCTCTTTGGAATGAA
CLDN10-R	ATCCCAGCCAAACAAGCAAT
CXCL16-F	CATCGGTTCAGTTCATGAATCG
CXCL16-R	GGAGCTGGAACCTCGTGTAGTATAG
EPSTI1-F	CAGAGGCGCACAAGTGCATA
EPSTI1-R	TCCTGCTCCGCAATTCTTTG
CDC42EP3-F	TGTCCTGGTTGTGGTCAAGACT
CDC42EP3-R	CCTGAGGTTACGGCCAAGTG
ZAN-F	TGTGGTTGCTATGGGTTTCATC
ZAN-R	GATACGGGAGGAAGCTCTGGTA
TCEA3-F	TTCTGCCTCCTCCTCTCCAA
TCEA3-R	GCTCTCCGCTTTTGATTTGC
PALMD-F	TTATGATGATGGGCAAAAGTCAGT
PALMD-R	CATCGGTGCCATTGTATGCT
RPL13A-F	TTGGACTTTCCACCTGGTCATAT
RPL13A-R	GTGTACAACAGCAAGCTCATGCT
GAPDH-F	CCAGGTGGTCTCCTCTGACTTC
GAPDH-R	GCTTGACAAAGTGGTCGTTGAG

To determine the concentration and quality of each cDNA sample, all cDNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Thermo). Q-PCR was performed using the Rotor-Gene 6000 multiplex system and data analysis conducted with the corresponding software version 1.7 (Corbett Research/Qiagen). For all genes except for AXIN2, in a 20 µl reaction: 300 nM of each primer, 120 ng of cDNA and 2X SYBR Green Mix (Bio-Rad) were added. Q-PCR cycling conditions were: initial denaturation at 95°C for 10 min; 45 cycles were divided into two steps, 10 cycles of 95°C for 20 s, 55°C for 40 s and 72 for 40 s and 35 cycles of 95°C for 30 s, 60°C for 60 s and 72°C for 30 s; final melt at 72–95°C rising by 0.2°C each step. The Q-PCR cycling conditions for AXIN2 differed from the other seven genes and were as follows: in a 20 µl reaction volume, 200 nM of each PCR primer, 2X SYBR Green Mix (Bio-Rad) and 80 ng of cDNA was added. Q-PCR cycling conditions were: initial denaturation at 95°C for 10 min; 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s; final melt at 72–95°C raising by 0.2°C each step. Each sample was performed in quadruplicate. A series of independent *t*-tests were used to examine the Q-PCR results and then compared to the microarray analysis data set. The same tissue comparisons were made as the microarray analysis and fold changes were calculated by a log equation ($2^{\wedge-\Delta\Delta Ct}$) as previously described in Haupt et al. [14].

Immunohistochemical analysis

For IHC analysis, 5 µm-thick, formalin-fixed paraffin-embedded sections were placed on aminopropyltriethoxysilane pre-coated slides, deparaffinized by xylene and rehydrated in graded ethanol and distilled water. For antigen retrieval, sections were placed in a BORG decloaker (pH 9.0; Biocare Medical, Concord, Australia) and heated in a pressure vessel at 125°C for 5 min. Sections were immunostained on the Labvision Autostainer 360 Autostainer (Thermo Scientific) using the Novolink DAB detection kit (Leica Microsystems), yielding a brown reaction product for AXIN2 staining. The AXIN2 antibody utilized was a rabbit polyclonal antibody obtained from Abcam (ab32197). Slides were treated with hydrogen peroxide for 5 min as an endogenous enzyme blocker and washed with PBS. Protein blocking reagent (Novolink kit) was applied and incubated for 5 min followed by air-drying of the slides. Sections were then treated with 2 µl/ml of the AXIN2 antibody for 120 min and washed in 1× PBS. Post primary reagent (Novolink kit) was then applied and incubated for 30 min and washed with PBS. Following this, polymer reagent (Novolink kit) was applied and slides incubated for 30 min then washed in 1× PBS. Sections were then treated with DAB stain for 2 min, washed with

distilled water and DAB stained for another minute. Slides were then washed with deionised water and counterstained with Mayer's hematoxylin. Sections were then dehydrated through an ethanol series and mounted with DPX (BDH Laboratory, Poole, England). Tissue sections were visualized under light microscopy (Leica Microsystems) and photographed using a ScanScope virtual microscopy system with the ScanScope program. Sections showing staining artefacts initially were repeated.

The immunostaining results for the tissue sections were graded in a semi-quantitative manner by determining the intensity of staining of each individual section and rated on a scale from 0 to 3. Briefly 0 = no staining; 1 = minimal/faint staining; 2 = moderate staining; and 3 = intense staining. Scoring was then carried out independently by two authors (P.N.G. and R.A.S.) and scores compared to ensure concordance. The percentage of ductal/nodal cells that were positively stained was scored as follows: a = 0% (score 0); 1–20% (score 1); 21–40% (score 2); 41–60% (score 3); 61–80% (score 4); or 81–100% (score 5). In total, 22 tumours were available for the full-section analysis (five grade 1; seven grade 2; six grade 3; and four control tissues). A series of independent *t*-tests and one-way analysis of variance (ANOVA) tests were used to compare scores between different histological groups as well as to other known factors like ER status, lymph-node status and in situ carcinoma status.

Results

Microarray analysis

From the array data, an independent group *t*-test revealed 178 genes significantly ($P \leq 0.01$) differentially expressed between the three grades of malignant breast tumours examined when compared to benign tissues. Six tumour group comparisons were made between the three grades and benign tumours where each comparison revealed a different number of significantly ($P \leq 0.01$) differentially expressed genes. Briefly: the comparison between grade I tumours and benign tissue revealed 24 genes; the comparison between grade II tumours and benign tissue revealed 14 genes; the comparison between grade III tumours and benign tissue revealed 22 genes; the comparison between grade I and II tumours revealed 64 genes; the comparison between grade I and III tumours revealed 20 genes; and the comparison between grade II and III revealed 34 genes. From these comparisons, an expression fold value was determined; with any fold changes below one deemed to be indicative of a down-regulation of gene expression and values above one indicative of an up-regulation of gene expression. From the six comparison groups, eight genes appeared in more than one comparison group and have been previously associated with processes associated with breast cancer.

The first gene, AXIN2 was significantly ($P = 0.003$) up-regulated (1.38-fold) between grade I and II tumours. This gene was also significantly ($P = 0.009$) down-regulated (0.758-fold) between grade III and control tumours. Finally, AXIN2 was significantly ($P = 0.003$) down-regulated (0.807-fold) between grade III and II tumours. Chemokine ligand 16 (CXCL16), the second gene of interest was significantly ($P = 0.00084$) down-regulated (0.674-fold) between grade I and control tissues. Comparisons between grade I and II were also significantly ($P = 0.0056$) down-regulated (0.740-fold) for this gene. CXCL16 was also significantly ($P = 0.0098$) down-regulated (0.760-fold) between grade II and control tissues. The third gene of interest was Claudin 10 which was significantly ($P = 0.00314$) up-regulated (1.381-fold) between grade I and control tissues. This gene was also significantly ($P = 0.0056$) up-regulated (1.344-fold) between grade I and II tumours. The fourth gene of interest was the epithelial stromal interaction 1 (EPSTI1) gene, which was significantly ($P = 0.0042$) up-regulated (1.355-fold) between grade I and control tissues. This gene was also significantly ($P = 0.0089$) up-regulated (1.30-fold) between grade I and II tumours. The gene CDC42 effector protein 3 (CDC42EP3) was significantly ($P = 0.006$) down-regulated (0.738-fold) between grade I and III tumours. This gene was also significantly ($P = 0.006$) down-regulated (0.734-fold) between grade II and III tumours. Zonadhesin (ZAN) was significantly ($P = 0.0087$) down-regulated (0.762-fold) between grade I and control tissues. This gene was also significantly ($P = 0.0075$) down-regulated (0.757-fold) between grade I and III tumours. Gene expression of transcription elongation factor A 3 (TCEA3) was significantly ($P = 0.0063$) up-regulated (1.481-fold) between grade I and II tumours. This gene was also significantly ($P = 0.0042$) down-regulated (0.657-fold) between grade II and III tumours. The final gene of interest, Palmdelphin (PALMD) was significantly ($P = 0.0095$) up-regulated (1.546-fold) between grade I and II tumours. This gene was also significantly ($P = 0.0068$) down-regulated (0.630-fold) between grade II and III tumours. Gene expression changes detected by the microarray are summarised in Fig. 1.

Q-PCR analysis

Q-PCR analysis of the identified eight genes of interest in the same population revealed the same trend in gene expression as demonstrated in the microarray analysis. Figure 2 summarises the fold changes between the

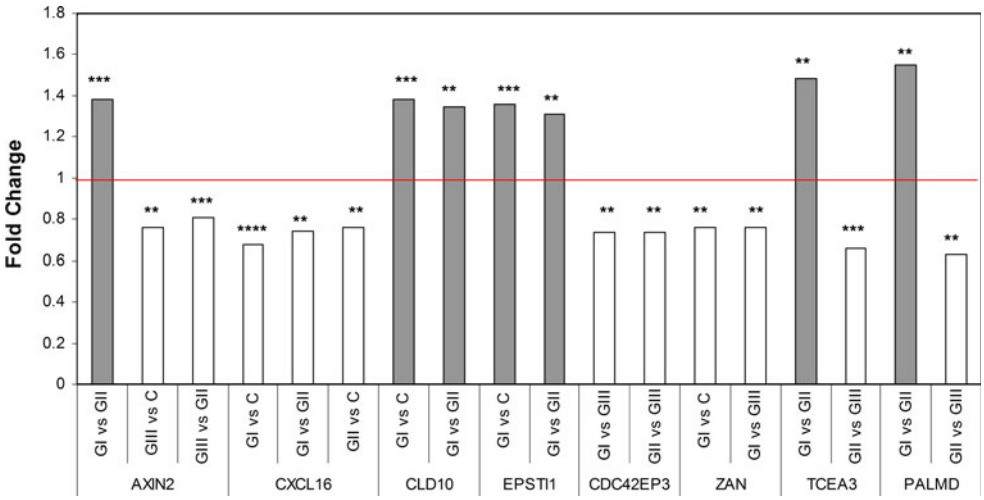

Fig. 1 Microarray results: a summary of tissue comparisons where gene expression differences between tissue comparisons were demonstrated to show significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$, ****$P \leq 0.001$). Up-regulated genes are above 1 fold change and shown in *grey* while down-regulated genes are below onefold change and shown in *white*

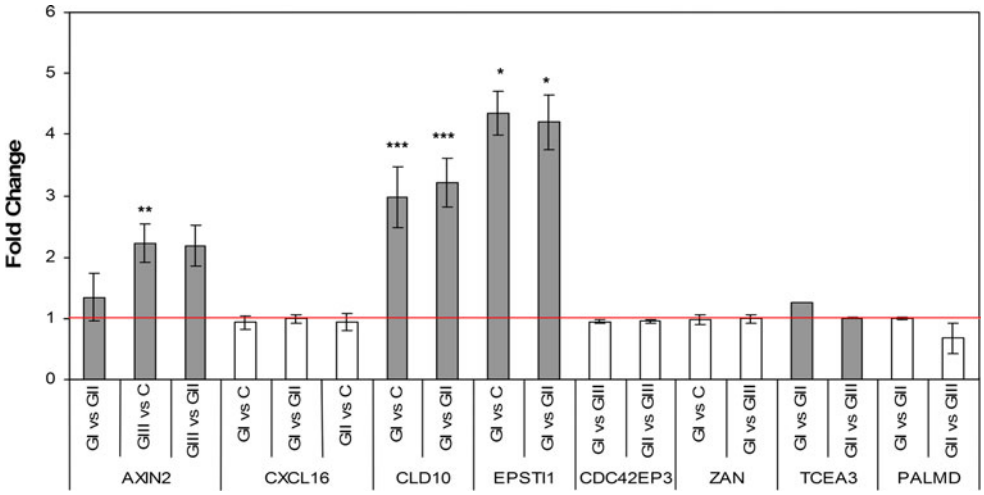

Fig. 2 Q-PCR results: a summary of tissue comparisons where gene expression differences between tissue comparisons were demonstrated to show significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$). Up-regulated genes are above onefold change and shown in *grey* while down-regulated genes are below onefold change and shown in *white*

microarray and Q-PCR analyses. Three of the genes (CLD10, ESPTI1 and AXIN2) demonstrated statistical significance. CLD10 was significantly ($P = 0.00426$) up-regulated (7.83-fold) between grade I and control tissues. The gene was also significantly ($P = 0.0024$) up-regulated (9.21-fold) between grade I and II tumours. ESPTI1 was significantly ($P = 0.0306$) up-regulated (20.31-fold) between grade I and control tissues. This gene was also significantly ($P = 0.0352$) up-regulated (18.36-fold) between grade I and III tumours. AXIN2 was significantly ($P = 0.00624$) down-regulated (0.394-fold) between grade III and control tissues. Changes in gene expression between grade I and II tumours for AXIN2 demonstrated an up-regulation of gene expression (1.34-fold) however this change was not significant ($P = 0.077$). There was also no significant ($P = 0.634$) difference between grade III and II tumours with a fold change of 1.01. CXCL16 also followed the same trend as the microarray analysis in all comparisons (GI vs. C, $P = 0.221$, 0.92-fold; GI vs. GII, $P = 0.605$, 0.988-fold; GII vs. Control, $P = 0.365$, 0.94-fold) however none showed any statistical significance. CDC42EP3 Q-PCR analysis also revealed a similar trend to the microarray results in both comparisons (GI vs. GIII, $P = 0.086$, 0.94-fold; GII vs. GIII, $P = 0.113$, 0.947-fold) however neither presented any significance below $P = 0.05$. Similarly, ZAN Q-PCR analysis followed the microarray analysis (GI vs. Control, $P = 0.816$, 0.971-fold; GI vs. GIII, $P = 0.94$, 0.99-fold) and no significant difference was seen at $P = 0.05$. TCEA3 and PALMD were particularly difficult to quantify using Q-PCR and not every sample was able to be amplified even after multiple runs, which was reflected in their lack of significance. TCEA3 produced mixed Q-PCR results where the comparison between grade I and II tumours was following the same trend as the microarray with a fold change of 1.25 ($P = 0.375$) however the comparison between grade II and III tumours did not follow the microarray trend with a fold change of 1.00 ($P = 0.596$), indicating no significant difference between the comparisons. Similarly, PALMD expression was insignificant between both comparisons (GI vs. GII, $P = 0.994$, 0.993-fold;

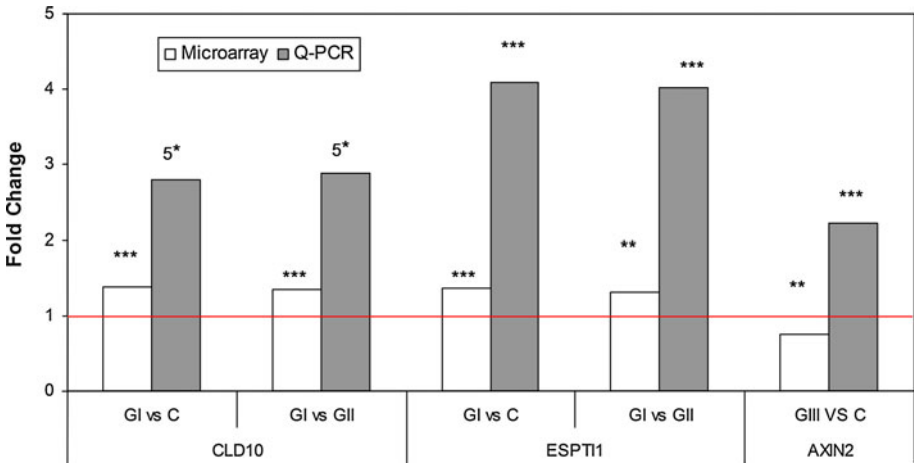

Fig. 3 Microarray analysis compared to the extended population Q-PCR results: a summary of tissue comparisons where gene expression differences between tissue comparisons were demonstrated to show significance (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$, 5*$P \leq 0.0005$). Up-regulated genes are above onefold change and shown in *grey* while down-regulated genes are below onefold change and shown in *white*

GII vs. GIII, $P = 0.247$, 0.678-fold) however the GII vs. GIII comparison followed the same trend as the microarray analysis which the GI vs. GII comparison did not, as shown in Fig. 3.

To determine if the modes of expression were consistent in breast tumours, a further seven samples (one grade I, three grade II, two grade III and one control) were also subjected to Q-PCR and analysed with the original 12 samples ($n = 19$). The three genes (CLD10, ESPTI1 and AXIN2) which showed statistical significance in the original samples were analysed and the trends in expression were consistent with those from the Q-PCR of the original samples. CLD10 was significantly ($P = 0.000242$) up-regulated (2.80-fold) between grade I and control tissues and significantly ($P = 0.00027$) up-regulated (2.89-fold) between grade I and II tumours. ESPTI1 was significantly ($P = 0.003$) up-regulated (4.09-fold) between grade I and control tissues and significantly ($P = 0.0033$) up-regulated (4.02-fold) between grade I and II tumours. AXIN2 was significantly ($P = 0.0037$) up-regulated (2.22-fold) between grade III and control tumours.

Immunohistochemistry

An example of the IHC staining of one grade III tumour using the AXIN2 antibody can be seen in Fig. 4. H&E sections were used phenotypically to localise staining to the epithelial tumour component of each sample. Generally all tissues stained well, with only two of the control tissues unable to be successfully stained, perhaps due to the high level of fat content in those particular samples, and as such these samples were excluded from further analysis. The remaining 20 tissues were analysed for signal intensity and percentage of staining. While the intensities varied amongst the grades, the percentages remained relatively constant (score 5 or 81–100%) within all tissues and subsequently this parameter was left out of the analysis. The average intensity scores of the tissues were as follows: controls = 2.25, grade I = 2.6, grade II = 2.356 and grade III = 2.041, which can all be seen in Fig. 5. A series of independent *t*-tests could not identify a significant difference between any of the six comparisons (GI vs. GII $P = 0.331$, GI vs. GIII $P = 0.090$, GI vs. C $P = 0.367$,

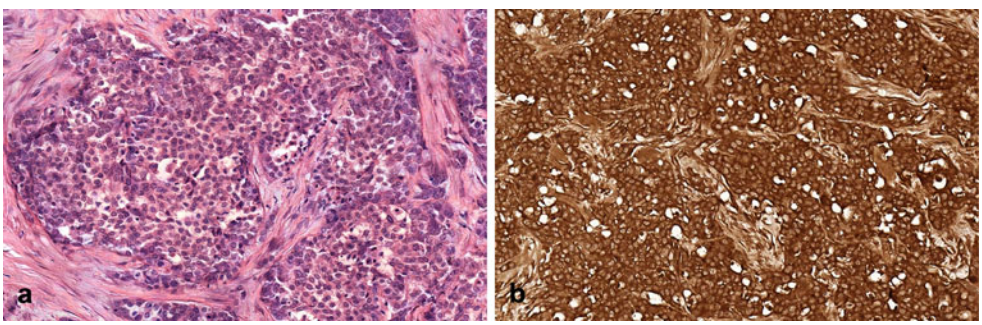

Fig. 4 Immunohistochemistry of AXIN2: **a** H&E staining of one grade III tumour, noting the heavily haematoxylin (*purple*) staining distinguishing the tumour from the eosin-stained (*pink*) stroma. H&E slides were used for each antibody-stained slide to view the position of the tumour versus the stroma. **b** AXIN2 staining of the same grade III tumour, noting the *darker brown* staining (intensity score = 2 [moderate]) of the nodal/ductal cells

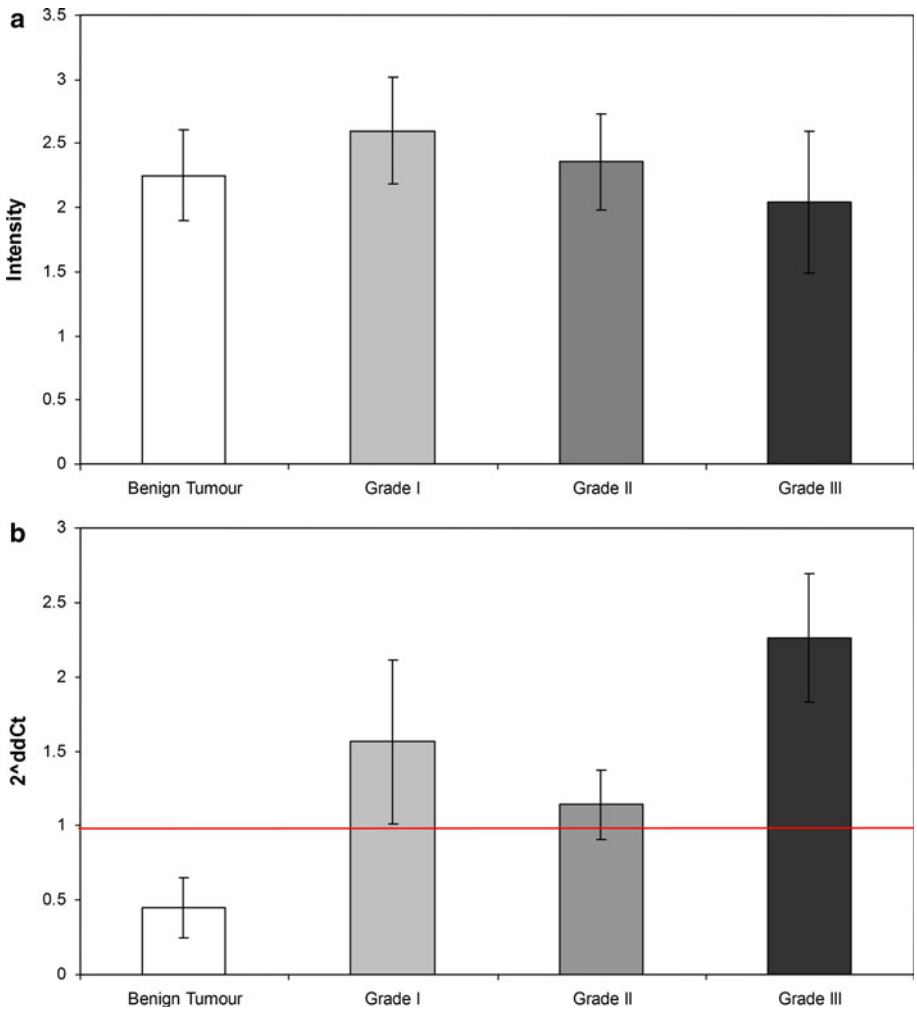

Fig. 5 Immunohistochemistry analysis of AXIN2: **a** The average intensity scores of the tissues. A series of independent *t*-tests could not identify a significant difference between any of the six comparisons (GI vs. GII $P = 0.331$, GI vs. GIII $P = 0.090$, GI vs. C $P = 0.367$, GII vs. GIII $P = 0.271$, GII vs. C $P = 0.750$ and GIII vs. C $P = 0.582$). **b** The relative gene expression of AXIN2 by Q-PCR of the original population where up-regulated genes are above onefold change while down-regulated genes are below onefold change

GII vs. GIII $P = 0.271$, GII vs. C $P = 0.750$ and GIII vs. C $P = 0.582$). The one-way ANOVA tests used to compare the intensity scores between different histological groups to other known factors such as ER status, lymph-node involvement and in situ carcinoma status revealed a significant effect between AXIN2 intensity and ER status where intensity is significantly ($P = 0.008$) higher in ER+ tumours compared to ER− tumours. The ANOVA analysis did not find a significant association between AXIN2 intensity and lymph-node status or in situ carcinoma status.

Discussion

A number of gene expression changes have been identified in human breast cancers including the establishment of the basal and luminal cell subtypes, demonstrating the promise for developing more personalised therapeutics. Microarray analysis of twelve samples of differing grades of breast cancer revealed eight genes involved in processes associated with the disease progression which were significantly ($P \leq 0.01$) differentially expressed (Fig. 1). Q-PCR analyses of these genes of interest in the same population revealed the same trend of gene expression to the microarray analysis (Fig. 2 fold changes between the microarray and Q-PCR analysis). Three of these genes also demonstrated statistical significance ($P \leq 0.05$) including CLD10, ESPTI1 and AXIN2. Immunohistochemistry analysis examining AXIN2 also showed a general (but not statistically significant) decline of protein expression with increasing tumour grade.

Up-regulated genes

CLD10 expression has not been previously widely associated with breast cancers. Previous studies have associated down regulation of CLD10 expression with a prolonged disease free period after curative surgery in hepatocellular carcinoma patients [15] along with localised expression of this gene in the kidney medulla, relative to cellular permeability [16]. In terms of the mammary gland, work by Qin et al. [17] demonstrated that CLD10 was associated

with mammary arteries. Interestingly, this study clearly demonstrated that normal coronary arteries have an entirely different array of genes expressed when compared to mammary associated arteries. This data implies that CLD10 over-expression may be associated with new blood vessel growth during breast tumour expansion and the mechanism is later lost or becomes less important to tumour survival.

Our microarray analysis revealed that Claudin 10 was significantly ($P = 0.00314$) up-regulated (1.381-fold) between grade I and control tissues and significantly ($P = 0.00314$) up-regulated (1.381-fold) between grade I and II tumour samples. In the Q-PCR analysis, CLD10 was significantly ($P = 0.00426$) up-regulated (2.96-fold) between grade I and control tissues and significantly ($P = 0.0024$) up-regulated (3.20-fold) between grade I and II tumours. In the extended Q-PCR population, CLD10 was also significantly ($P = 0.000242$) up-regulated (2.80-fold) between grade I and control tissues and significantly ($P = 0.00027$) up-regulated (2.89-fold) between grade I and II tumours. This pattern of expression may indicate early tumour cells increase CLD10 expression to mediate connections with nearby vascular associated stromal cells to increase extracellular signalling. With direct cell adhesion no longer required for tumour survival, the subsequent decrease of CLD10 expression as tumour grade increases, may further demonstrate reduced cell adhesion in preparation for motility and potential metastasis. The consistency of the microarray and Q-PCR analyses, along with the demonstrated statistical significance of the expression data make CLD10 an excellent candidate for further investigation for its role in human breast tumours, particularly lower grade tumours with metastatic potential.

Morphogenesis can be defined as development through growth and differentiation. According to Gouon-Evans et al. [18] epithelial/mesenchymal cell interactions are a necessary process in normal ductal morphogenesis throughout all stages of mammary gland development. The breakdown of epithelial cell homeostasis resulting in cancer progression has been correlated with the loss of epithelial characteristics and the acquisition of a migratory phenotype [19]. Our microarray analysis identified that the Epithelial stromal interaction 1 (EPSTI1) gene, which has a central role in morphogenesis, was significantly ($P = 0.0042$) up-regulated (1.355-fold) between grade I and control tissues and significantly ($P = 0.0089$) up-regulated (1.30-fold) between grade I and II tumours. Q-PCR analysis validated this expression profile, demonstrating significant ESPTI1 ($P = 0.0306$) up-regulation (4.34-fold) between grade I and control tissues and significant ($P = 0.0352$) up-regulation (4.20-fold) between grade I and III tumours. In the extended Q-PCR population, ESPTI1 was significantly ($P = 0.003$) up-regulated (4.09-fold) between grade I and control tissues and significantly ($P = 0.0033$) up-regulated (4.02-fold) between grade I and II tumours.

The up-regulation of EPSTI1 in grade I tissues when compared to normal tissues is slightly higher than that between grade I and II, indicating an expression increase during the transformation of normal cells to tumour cells. This was an unexpected trend in gene expression as deregulation of morphogenesis associated genes would be expected as grade increases. This suggests that as the epithelial cells become more disregulated and increase in tumour grade, the more they express this morphogenic gene i.e. a marker of change. In invasive breast cancers, tumour cells are in direct contact with the surrounding highly activated collagenous stroma following basement membrane degradation. In support of this hypothesis, EPSTI1 has been demonstrated to play a role in the interaction of tumour cells with connective tissue fibroblasts [20]. The study by Gudjonsson et al. [20] found EPSTI1 gene expression highly up-regulated in a series of breast carcinomas when compared with normal breast tissue. This study, along with another by Petersen et al. [21] suggests that breast cancers can generate their own non-malignant supportive stroma by interacting with epithelial tumour cells whilst facilitating tumour growth. These studies align well with our findings suggesting the role of EPSTI1 extends beyond morphogenesis toward epithelial-stromal communication and subsequent signalling events. Similarly to CLD10, the consistency between the microarray and Q-PCR analyses along with the statistical significance implicate EPSTI1 as an excellent candidate for further investigation for its role in human breast tumour progression.

Down-regulated genes

Chemokine ligand 16 (CXCL16) is not only involved in chemotaxis but also in receptor mediated endocytosis [22], adhesion [23] and known to be involved with LDLR activity [24]. In a murine model, use of an adenovirus encoding CXCL16 has been demonstrated to inhibit mammary tumour growth and prevent metastases following surgical removal of the primary tumour [25]. This study also demonstrated significant inhibition of metastases following infection with adenovirus CCL16 resulting in complete tumour ablation in up to 63% of treated mice [25]. Another chemokine, the CXCR4 receptor and its unique ligand, CXCL12 (stromal cell-derived factor-1), have also been implicated in cancer metastasis. Several studies have demonstrated that CXCR4 and CXCL12 regulate tumour cell metastasis to specific organs including the prostate [26, 27]. Previous work has also demonstrated that CXCL12 enhances prostate cancer cell adhesion,

migration and invasion implicating ligand and receptor in prostate cancer metastasis [26]. These studies support a role for chemokines and their ligands in metastasis as the cancer grade increases.

Our microarray analysis revealed that CXCL16 gene expression was significantly ($P = 0.00084$) down-regulated (0.674-fold) between grade I and control tissues. Comparisons between grade I and II tumours also demonstrated a statistically significant ($P = 0.0056$) down-regulation (0.740-fold) for this gene. The expression of CXCL16 was also significantly ($P = 0.0098$) down-regulated (0.760-fold) between grade II tumours and control tissues. On two of the three grade comparisons, CXCL16 gene expression was demonstrated to be up-regulated in control tissues, indicating that this gene may be acting as a tumour-suppressor. Validation analysis by Q-PCR demonstrated the same trend in gene expression of CXCL16 in all comparisons (GI vs. C, $P = 0.221$, 0.92-fold; GI vs. GII, $P = 0.605$, 0.988-fold; GII vs. Control, $P = 0.365$, 0.94-fold) however none were of statistical significance. At first glance, the down-regulation of CXCL16 in both grade I and II tumours was unexpected, however on closer examination, fold change values become smaller as grade increases. As tumour grade increases, the level of gene expression down-regulation is reduced, i.e. closer to the level observed in the control tissues. Since this gene is a receptor ligand, it is possible that this loss of expression relates to tumour cell evasion of immune surveillance. While both microarray and Q-PCR demonstrated consistent trends in expression of this gene, levels of expression were not statistically significant via Q-PCR. However, the involvement of this gene in chemotaxis and other cancer-related processes make it an ideal candidate for further investigation within less aggressive and metastatic tumours and more particularly benign dysplasia tumours.

CDC42 effector protein 3 (also known as CEP3) is part of the CDC42EP family of proteins which act downstream of CDC42 to induce actin filament assembly resulting in cell shape changes [28]. CDC42EP3 mRNA is thought to be a target of PUMILIO2 protein in the human male gonad and to be under translational control mediated by specific nucleotide motifs within the 3′UTR in human reproduction [29]. Our microarray analysis demonstrated that gene expression of CDC42EP3 was significantly ($P = 0.0067$) down-regulated (0.738-fold) between grade I and III tumours and significantly ($P = 0.006$) down-regulated (0.734-fold) between grade II and III tumours. Q-PCR validation of gene expression analysis revealed a similar trend to the microarray results in both comparisons (GI vs. GIII, $P = 0.086$, 0.94-fold; GII vs. GIII, $P = 0.113$, 0.947-fold) however neither presented any significance below $P = 0.05$.

From the data, expression of CDC42EP3 is slightly higher in grade I tumours when compared to grade II tumours, with a rapid increase in gene expression in grade III tumours. Typically tumour progression is associated with a loss of regulation of cell shape however, other factors and/or genes likely influence the expression of CDC42EP3 and its up-regulation. As actin is not only involved in filament assembly but is also a motility protein which interacts with myosin, the increase observed in grade III tumours may be associated with the tumours' metastatic potential. Like CXCL16, microarray and Q-PCR analyses demonstrated consistent trends of CDC42EP3 gene expression. Association of this gene with cancer is relatively undocumented, but data here implicated this gene as an ideal candidate for further investigation in more aggressive and metastatic tumours, particularly grade III tumours.

Zonadhesin (ZAN) is a male reproductive protein localised on the sperm head, comprising many domains known to be involved in cell–cell interaction or cell adhesion [30]. Microarray analysis revealed that ZAN gene expression was significantly ($P = 0.0087$) down-regulated (0.762-fold) between grade I and control tissues and significantly ($P = 0.0075$) down-regulated (0.757-fold) between grade I and III tumours. Analysis of ZAN by Q-PCR followed a similar trend in the gene expression profile (GI vs. Control, $P = 0.816$, 0.971-fold; GI vs. GIII, $P = 0.94$, 0.99-fold), however this was not statistically significant. The expression of ZAN was lowest in the grade I and highest in the grade III tumour samples examined. As cell adhesion is reduced during cancer progression, leading to metastasis, here we have observed an expected and unexpected result. We observed that regulation decreases with tumour progression of normal cells into grade I cells which is expected and that regulation increases with tumour progression of a grade I cell into a grade III cell which is unexpected as regulation was shown to be lowest in grade I.

Other studies have found that haplotypes segregating with a frame-shift mutation suggest that ZAN is a potential pseudogene [30]. It is possible that the expression of ZAN in normal cells decreases as they transform into grade I tumour cells, with a continued decrease in expression in grade II tumours, and a subsequent increase in expression in grade III tumours. This may be a result of the postulated frameshift mutation, resulting in altered function and the role of a potential pseudogene. Like the other two down-regulated genes CXCL16 and CDC42EP3, our microarray and Q-PCR showed consistent trends of expression, however they proved to be not significant in the Q-PCR analysis, however this gene's involvement in cell–cell interaction and cell adhesion warrant further investigations

amongst breast tumours of all grades, especially grade III and benign breast tumours.

Up and down-regulated genes

Transcription elongation factor 3 (TCEA3, TFIIS) promotes efficient elongation by RNA polymerase II (RNAPII) [31]. In vitro, TCEA3 stimulates RNAPII stalled at a block to elongation to cleave the nascent transcript and subsequently resume elongation. There is also an association between TCEA3 and elongating RNAPII under conditions of stress in an in vivo model [32]. Transcript elongation by RNA polymerase is a dynamic process, responsive to a number of intrinsic and extrinsic signals [31]. TCEA3 has been identified to enhance the rate or efficiency of transcription and to facilitate RNA polymerase transcription through blocks to elongation by stimulating the polymerase to cleave the emerging RNA transcript within the elongation complex [31]. However, while the regulation of these factors can be seen between the different grades, their affects on subsequent target genes is unknown.

In our microarray analysis, TCEA3 was significantly ($P = 0.0063$) up-regulated (1.481-fold) between grade I and II tumours and significantly ($P = 0.0042$) down-regulated (0.657-fold) between grade II and III tumours. TCEA3 was particularly difficult to quantify using Q-PCR and not every sample was able to be amplified even after multiple runs. TCEA3 produced mixed Q-PCR results where the comparison between grade I and II tumours was following the same trend as the microarray with a fold change of 1.25 ($P = 0.375$) however the comparison between grade II and III tumours did not follow the microarray trend of gene expression with a fold change of 1.00 ($P = 0.596$), indicating no significant difference between the grade comparisons. Our findings indicate a departure from up-regulation in grade II and strong up-regulation in grade I and even higher in grade III. In breast cancer it is expected that transcription factors are up-regulated as tumour grade increases. It is possible that other interacting genes may be influencing TCEA3 expression in grade II which would explain its low abundance in this tumour grade. It may be that other genes may be taking up the role of TCEA3 during the particular biochemical stimulations occurring in the grade II tumours or that an increased level of expression changes associated with the transition from normal to grade I tumour and with the transition from a tumour cell to an invasive or metastatic (grade III) tumour cell. The mixed consistency of the microarray and Q-PCR results could be attributed to an error in the microarray, since this gene was particularly difficult to investigate using Q-PCR. However TCEA3's involvement in transcription and RNA elongation, merit further investigation of this gene and its association with breast tumours, particularly grade II and III breast tumours.

Palmdelphin (PALMD) is a newly identified cytosolic isoform of paralemmin-1, a lipid raft-associated protein implicated in cell shape control [33]. Studies into the processes and interactions of PALMD have demonstrated that the cell shape regulator behaves as a cytosolic protein which can be partially recruited from the cytosol to the detergent-resistant fraction of a membrane/cytoskeletal cell ghost preparation, which suggest that PALMD may peripherally associate with endomembranes or cytoskeleton-linked structures [33]. Our microarray analysis revealed that PALMD gene expression was significantly ($P = 0.0095$) up-regulated (1.546-fold) between grade I and II tumours and significantly ($P = 0.0068$) down-regulated (0.630-fold) between grade II and III tumours. PALMD, like TCEA3, was particularly difficult to quantify using Q-PCR and not every sample was able to be amplified even after multiple runs. Using Q-PCR validation, expression of PALMD was not statistically significant between both comparisons (GI vs. GII, $P = 0.994$, 0.993-fold; GII vs. GIII, $P = 0.247$, 0.678-fold) however the GII versus GIII comparison followed the same trend as the microarray analysis.

Palmdelphin, like CDC42EP3 discussed earlier is also a regulator of cell shape. Like CDC42EP3, PALMD follows the same pattern of expression. Microarray analysis indicated that PAMLD expression was high in grade I, reduced in grade II and becomes slightly higher than grade I in the grade III tumours examined. Similarly, CDC42EP3 gene expression was highest in grade III tumours and lower in grade I and II tumour samples examined. This may be a common pattern with genes associated with cell shape regulation. The reduced level of gene expression in grade II tumours is of potential interest for a role for these genes in progression toward more aggressive and potentially invasive tumours or during a tumour's expansion and progression. The role of the genes involved in cell shape, like CDC42EP3 and PALMD may have a role in metastasis and warrant further investigations amongst breast tumours of all grades.

AXIN2, a homologue of AXIN1, encodes a protein with 60% amino acid identity to AXIN1 and both proteins contain the same putative conserved domains for binding to APC, GSK3β, CK1, and β-catenin. Although tissue distribution and transcriptional regulation of AXIN1 and AXIN2 are significantly different, the two proteins are functionally equivalent in vivo [34]. The AXIN2 gene has been mapped to 17q23-q24 which is a region that shows frequent loss of heterozygosity (LOH) in breast cancer, neuroblastoma and other tumours [35]. As AXIN2 can inhibit β-catenin abundance and function, it is postulated that AXIN2 may regulate Wnt signalling in a negative

feedback pathway. Additionally, although AXIN1 and AXIN2 are thought to have comparable functions, the observation that Wnt pathway activation elevates AXIN2 but not AXIN1 expression suggests that there may be significant functional or pathway divergence between these two highly homologous proteins [36].

Microarray analysis demonstrated AXIN2 gene expression significantly ($P = 0.003$) up-regulated (1.38-fold) between grade I and II tumours and significantly ($P = 0.009$) down-regulated (0.758-fold) between grade III and control tumours. AXIN2 was also significantly ($P = 0.033$) down-regulated (0.807-fold) between grade III and II tumours. In the Q-PCR analysis, AXIN2 was significantly ($P = 0.00624$) up-regulated (3.51-fold) between grade III and control tissues. Changes in gene expression between grade I and II tumours for AXIN2 reflected an up-regulation (1.34-fold) however this change was not statistically significant ($P = 0.077$). There was also no significant ($P = 0.634$) difference between grade III and II tumours with a fold change of 1.01. In the extended Q-PCR population, AXIN2 was significantly ($P = 0.0037$) up-regulated (2.22-fold) between grade III and control tumours. The inconsistency of regulation for AXIN2 between grade III and control tissues between methods is perhaps explained by the cluster analysis performed on the microarray samples in our previous publication [12] where AXIN2 expression was identified to be consistently up-regulated as grade increases, to be expected of a negative regulator of Wnt signalling in breast cancer [37]. Although there is an apparent discrepancy between the initial microarray analysis and the Q-PCR data, the increased accuracy of Q-PCR highlights the need for the validation of microarray findings with Q-PCR analyses. It is possible that an up-regulated AXIN2 may still exert a level of growth regulation in cancers, but perhaps not to a level sufficient to completely arrest growth. Alternatively, competing proteins may be being interfering with or modulating some of its functions. Further investigations into the exact role of AXIN2 in breast cancer development are needed in all breast cancers of all grades.

Immunohistochemistry of AXIN2

As AXIN2 was altered in every tumour grade, we decided to investigate the expression of the protein by IHC. Twenty tissues were analysed for intensity and percentage of staining. The percentage of cells positive for AXIN2 showed little variability (score 5 or 81–100%) within all tissues. However, the average signal intensity scores of the tissues (Fig. 5a) showed a trend toward increasing signal intensity with tumour progression. A series of independent t-tests did not identify a significant difference between the six grade comparisons (GI vs. GII $P = 0.331$, GI vs. GIII $P = 0.090$, GI vs. C $P = 0.367$, GII vs. GIII $P = 0.271$, GII vs. C $P = 0.750$ and GIII vs. C $P = 0.582$). The relative gene expression of *AXIN2* using Q-PCR (Fig. 5b), demonstrated a lower level of gene expression in grade II tumours but at a higher level than the benign tumours examined. When comparing the relative expression of *AXIN2* to the staining intensity of AXIN2, it is apparent that the expression trend is lost between the mRNA and the protein stage and that AXIN2 is decreased as grade increases instead. This may represent miRNA repression or post-translational effects of AXIN2, decoupling the mRNA and protein results.

However a significant association was revealed by the one-way ANOVA test which showed a significant association between AXIN2 intensity and ER status where intensity is significantly ($P = 0.008$) higher in ER+ tumours compared to ER− tumours. This was a somewhat expected result given the data available for grades as AXIN2 intensity was greatest in grade I, composed entirely by ER+ tumours, and tumours typically loose ER+ status as they transform into tumours of higher grades. This was also the case for our grade III tumours which were all ER− and where AXIN2 staining intensity was the lowest. Yet to our knowledge this association has not previously been published. This presents a novel finding in AXIN2 protein expression and its association with ER status.

Conclusion

The objective of this research was to uncover potential relationships between gene expression and prognostic indicators of human breast cancers of specific and differing grades. This was accomplished through whole genome microarray analysis and the subsequent investigation of identified genes associated with processes involved in breast cancer and breast tumours of grades I–III including benign tumours. The clarification of these factors may provide relationships which can be used for the development of genetic tests to provide doctors and patients with more comprehensive information on the threat posed by specific tumours, leading to more individualised therapeutics.

From this study, the two most implicated candidates to have a role in less aggressive breast tumours, namely grade I tumours, were CLD10 and ESPTI1. Their gene expression profile from the microarray analysis was not only replicated by Q-PCR of the original tumour samples, but continued to demonstrate a similar significant trend of gene expression in our extended sample population. This study, along with the current literature available on both genes, presents both as possible indicators of less aggressive (grade I) human breast tumours. AXIN2 was not only

involved in all cancer grades on the RNA level but also at the protein level as indicated by IHC analysis. Using Q-PCR, the grade III to control tissue proved to have a significant ($P = 0.0062$) difference in AXIN2 gene expression. The IHC for AXIN2 also revealed increased staining intensity from benign tumours to grade I tumours, with a steady decrease in signal intensity with increasing tumour grade. A significant association of AXIN2 protein expression and ER status was also demonstrated, which when further validated, appears to be novel. CXCL16, CDC42EP3 and ZAN have not been widely investigated, and while our microarray and Q-PCR analyses implicated them in grade III breast tumours (CDC42EP2), benign tumours (CXCL16) or both (ZAN), these changes were not statistically significant when validated by Q-PCR analysis. While other chemokines have been associated with human cancers, the link between CXCL16 and breast cancer is yet to be elucidated. Although the significant TCEA3 and PALMD level of gene expression in the microarray data was not fully replicated in validation Q-PCR analysis, the roles of these genes in transcription and cell shape respectively, especially in grade III breast tumours warrant further investigation for their involvement in highly metastatic and invasive breast tumours.

Six of the seven genes (TCEA3 showed both up and down regulation between comparisons) identified using both microarray and Q-PCR validation were found to have reduced expression in the grade II tumours examined, suggesting that the largest change in the gene expression profile of tumours occurs during the transformation between grade II to grade III tumours. For Q-PCR validation in this study we chose a fold change value of $+/-1$ change as an indication of significant expression change. Although a value of 1.5 fold change is more commonly used, in this study using our criteria, we achieved and validated targets that clearly demonstrated consistent gene expression differences between control tissues and more aggressive breast cancers. The discrepancies between the Q-PCR and microarray experiments in this study may be due to a number of factors. The bias in the replication rounds required to obtain sufficient RNA for microarray analysis is a potential source for this. In addition, degradation of initial RNA from the FFPE tissue may have resulted in bias for or against certain transcripts, though this may also be a consequence of the generally high rate of variance in microarray results due to limitations in their construction. The two factors may also aggravate one another, with the individual biases leading to highly distorted expression estimates for particular genes. It is also possible that alternative splice variants of the genes that were able to be recognised by the probe-based system of the microarray may not have been sufficiently replicated in the Q-PCR analyses. However, the hierarchical cluster analyses would appear to have reliably categorised the samples into their respective grades, so such distortions may be limited to a relatively small proportion of genes. While these results do demonstrate that FFPE tissues can be useful in examining breast cancers, they also illustrate the need for appropriate experimental controls and replication along with subsequent validation of array data by Q-PCR or other methods.

From our study, we can conclude that there are significant differences in gene expression between different breast cancer grades and that expansion of this approach, especially using narrowed pathway specific approaches may lead to an improved ability to discriminate between cancer grade and other pathological factors. 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.

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