DNA Microarray-based Transcriptomic Profiling of an Isogenic Cell Culture Model of Breast Tumour Cell Invasion

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Abstract. *Background: The process of tumour invasion and subsequent metastasis represents the most lethal aspect of cancer and is responsible for the majority of deaths among cancer patients. Our objective was to identify invasion-specific genes in a series of isogenic breast cancer cell lines of differing invasive potential using DNA microarray-based transcriptomic profiling. Materials and Methods: A panel of increasingly invasive breast cancer subclones generated from the primary breast cancer cell line, Hs578T, were subjected to DNA microarray analysis using the Affymetrix HG-U133A array. Results: Data analysis using GeneSpring 6.0 generated a list of 508 differentially expressed genes. A total of 71 genes were down-regulated and 437 genes were up-regulated. Array data for 17 genes was validated using quantitative real-time RT-PCR analysis. A correlation coefficient of 0.9249 was obtained when both technology platforms were compared. Conclusion: Potential new biomarkers for the diagnosis and treatment of invasive breast cancer have been identified.*

Although tremendous progress has been made in the diagnosis and treatment of cancer over the last decade, the majority of patients die because their tumour has metastasised to other sites in the body (1). In order for cells to metastasise, they must first detach from the primary tumour, invade through the surrounding tissue and basement membrane, intravasate into the circulatory system and extravasate into distant organs (2). Therefore, a major challenge facing the cancer scientist is the development of improved methods to predict the metastatic potential of a patient's individual tumour and prevent local metastasis.

With the advent of DNA microarray technology, it is now possible to look globally at gene expression across the genome simultaneously, under identical conditions and to find patterns of expression. Elucidating patterns of gene expression should lead to a better understanding of the complex interactions between genes and how this ultimately impacts on the pathology of disease (3). DNA microarray analysis has been used to investigate the underlying biology of many cancer types, including breast cancer (4, 5). This type of analysis can be applied to every aspect of the disease including initiation, progression, invasion and drug resistance with the aim of elucidating the underlying molecular mechanisms involved (6). There are many published studies using both breast cancer cell lines (7) and breast tumour tissue (8) which have used DNA microarrays in an attempt to develop molecular portraits, classifications and prognostic signatures of breast cancer (5). An example of the potential for DNA microarrays in the clinical management of patients with cancer is the 70-gene prognostic breast cancer signature reported by van 't Veer *et al.* (9), which is now undergoing evaluation in clinical trials.

Using the primary human breast cancer cell line, Hs578T, we have previously established and extensively characterised a series of invasive subclones with different levels of invasion (submitted for publication by Hughes L, Malone C, Burger AM and S McDonnell, 2006). This cell line panel was subjected to DNA microarray analysis on the Affymetrix GeneChip platform and 508 differentially expressed genes identified. Altered expression of 17 genes was validated using quantitative real-time RT-PCR analysis. The genes identified in this study are potential new biomarkers of aggressive tumour behaviour and may be important in the diagnosis, prognosis and treatment of metastatic breast cancer.

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Materials and Methods

Cell lines and cell culture. The human breast cancer cell line Hs578T was obtained from the ATCC (Manassas, VA, USA). Four invasive subclones of this cell line referred to as Hs578Ts(i)$_2$, Hs578Ts(i)$_4$, Hs578s(i)$_6$ and Hs578Ts(i)$_8$ were isolated using selection through the BD Matrigel™ Invasion Chamber assay system. Cells were grown in

Table I. *List of primers used in quantitative real-time RT-PCR analysis.*

Primer	Primer sequence	Product size (base pair)	Annealing temperature ˚C
β-actin	P1 5' ATC CTG CGT CTG GAC CTG GCT 3'	554	55
	P2 5' CTT GCT GAT CCA CAT CTG CTG 3'		
Calponin 1	P1 5' TAG GTC CAC AAG GCC TTC AC 3'	238	57
	P2 5' GTT GGG GAC ACC CTG TTC TA 3'		
Cathepsin Z	P1 5' CTG GAT TGT CCG GAA TTC AT 3'	211	55
	P2 5' CAT AGG ATC CCC TCT GGT CA 3'		
CTAG1B	P1 5' CTC CAT CAG CTC CTG TCT CC 3'	209	57
	P2 5' CTC CTC CAG CGA CAA ACA AT 3'		
CYBA	P1 5' CGC TTC ACC CAG TGG TAC TT 3'	200	58
	P2 5' GAG AGC AGG AGA TGC AGG AC 3'		
Decorin	P1 5' GAA GGG AGA AGA CAT TGG TTT G 3'	193	55
	P2 5' GCA GAG GGG TAA ATT GAA ACA 3'		
IGFBP3	P1 5' AGG GCA CTC TGG GAA CCT AT 3'	166	56
	P2 5' TGA TGC CTC TGA ATG TGG AG 3'		
IGFBP5	P1 5' TAG CCC ACA TGG ATA GCA CA 3'	190	60
	P2 5' GGA GAG AAC AGA GTT ATG GTA TGA A 3'		
Keratin 18	P1 5' CAC AGT CTG CTG AGG TTG GA 3'	164	58
	P2 5' GAG CTG CTC CAT CTG TAG GG 3'		
Lumican	P1 5' GCA TGG AAA TCA GCC AAG TT 3'	163	52
	P2 5' TTG CAA TAT TCT TGG CCT CA 3'		
LYN	P1 5' CCA CAT TCG GGG CTA TTT TT 3'	150	54
	P2 5' AAC AGA ACC GCT TGA ACC TC 3'		
MMP-2	P1 5' CTG AGA TCT GCA AAC AGG ACA TTG T 3'	474	54
	P2 5' CCG CCC TGC AGG TCC ACG ACG GCA T 3'		
PCOLCE2	P1 5' TGC TGT CAG AGGAGC AGC TA 3'	210	55
	P2 5' GCA AAG GCT TGA AGG TGA AA 3'		
Pirin	P1 5' TGG AGA ACA AGG ATC CCA AG 3'	160	53
	P2 5' GCC CTT TCA AAC CCA TTT TT 3'		
PLAC8	P1 5' CTG CGG AGT CTG TCT CTG TG 3'	238	60
	P2 5' GAA AGT ACG CAT GGC TCT CC 3'		
S100A4	P1 5' GAT GAG CAA CTT GGA CAG CA 3'	155	56
	P2 5' CCC AAC CAC ATC AGA GGA GT 3'		
SERPINB2	P1 5' GAT GAG CTG TGT GCC TCA GA 3'	188	55
	P2 5' GGG CAT GAT CCC ATT TTA GA 3'		
Sulfatase 1	P1 5' GCT CCC CAA GAT GAT GTG TT 3'	215	55
	P2 5' CAC ATC AGG CAA GCA GCT AA 3'		
TNFRSF 11B	P1 5' TGC TTG CAG TAA TTC AAC TGG 3'	246	59
	P2 5' GTC ATA CCG TTT CTT TAT AGG ATG A 3'		

Dulbecco's Modified Eagle's Medium (Sigma, Dublin, Ireland) supplemented with 10% foetal bovine serum (FBS), 5% L-glutamine, 100 units/mL penicillin/streptomycin and 0.1unit/mL bovine insulin. Cells were cultured in a humidified incubator at 37˚C and 5% CO_2.

Invasion assay. Cells in serum-free media were seeded in triplicate into a 24-well (8 μm pore size) BD Matrigel™ Invasion Chamber (BD Biosciences, Europe). Chambers were placed into 24-well companion plates containing 20% FBS as the chemoattractant. Plates were incubated for 22 h at 37˚C with 5% CO_2 and then media plus cells removed from the top of the chamber using cotton swabs. The number of invading cells was determined by fixing the membrane in 10% formalin and staining with 2.5% crystal violet. The crystal violet stain was eluted from the cells with glacial acetic acid and the absorbance read at 620 nm.

DNA microarray analysis. RNA was extracted (in duplicate) from the five cell lines using TriReagent (Sigma) as per the manufacturer's instructions. RNA was purified using sodium acetate precipitation and double-stranded cDNA synthesis undertaken using the SuperScript Choice system and protocol (Invitrogen, BioSciences, Dublin, Ireland). Biotin labelling was undertaken using the Enzo BioArray™ Highyield™ RNA transcript labelling kit. RNA quality was checked using an Agilent Bioanalyzer and samples were fragmented prior to hybridization onto Human Genome U133A (HG U133A) GeneChip, as per established protocols in the Transcriptomics Facility of the UCD Conway Institute. DNA microarray data was analysed using GeneSpring 6.0 software.

Quantitative real-time RT-PCR analysis. Single-stranded cDNA was prepared from RNA extracted from 3 separate biological replicates per cell line. The reverse transcribed material was then amplified

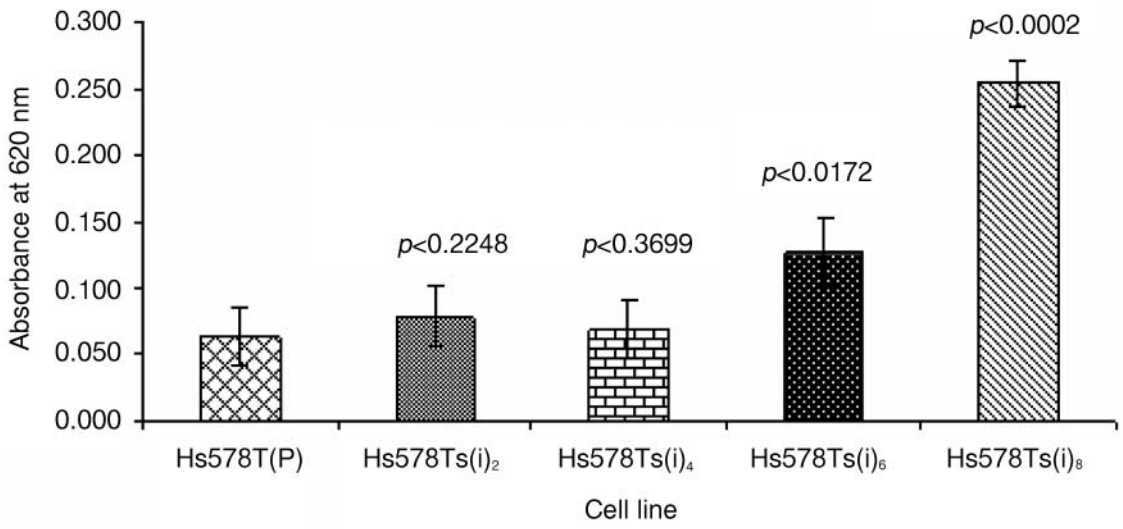

Figure 1. *Invasion assay of the parental cell line, Hs578T(P), and a series of invasive sublines, Hs578Ts(i)$_2$, Hs578Ts(i)$_4$, Hs578Ts(i)$_6$ and Hs578Ts(i)$_8$. A total of 1x10^5 cells were added to each well of 24-well invasion chambers and incubated for 22 h. Cells which had invaded were fixed, stained with crystal violet and stain eluted with acetic acid and absorbance read at 620 nm.*

by PCR using the primer sets indicated in Table I, which were designed using Primer 3 software. For the PCR reactions, SYBR Green real-time PCR master mix (Applied Biosystems, Warrington, England) and the ABI 7900HT system (Applied Biosystems) were used. Each sample was replicated without cDNA (no template controls) and at the end of the run, dissociation curves were performed at 90˚C to ensure only one product had been formed in each reaction. Resultant real time data was analysed using the delta delta CT method (10).

Results

Invasive activity of the Hs578T parental cell line and a series of invasive subclones. The Hs578T parental cell line, referred to as Hs578T(P), was subjected to several rounds of selection using the BD Matrigel™ Invasion Chamber assay system. Briefly, the parental cells were allowed to invade for 5 h and then invading cells collected from the underside of the membrane, expanded in culture and designated Hs578Ts(i)$_1$. Further rounds of selection generated a series of invasive subclones. After 8 rounds of selection, an invasive variant referred to as Hs578Ts(i)$_8$ was generated. This variant was 3-fold more invasive than the parental cell line *in vitro* and also displayed increased growth rate as a monolayer and in soft agar, as well as tumourigenicity *in vivo* (submitted for publication by Hughes L, Malone C, Burger AM and McDonnell S, 2006). The invasive activity *in vitro* of the parental cells and a series of subclones isolated after 2, 4, 6 and 8 rounds of selection is shown in Figure 1. The Hs578Ts(i)$_2$ and Hs578Ts(i)$_4$ showed fluctuating levels of increased and decreased invasiveness relative to the Hs578T(P). Figure 1 shows that it was not until round 6 (Hs578Ts(i)$_6$) of selection that a significant ($p<0.0172$) increase in invasiveness was observed. The invasive variant Hs578Ts(i)$_8$ was significantly ($p<0.0002$) more invasive than the parental cell line.

Affymetrix HG-U133A GeneChip analysis. Following RNA extraction, reverse transcription, cDNA synthesis and labelling, the cRNA was fragmented and a portion of each sample applied to Affymetrix test chips. The test chips contained the hybridisation controls bioB, bioC, bioD and cre. The test arrays also contained the 3' and 5' control probe sets for the housekeeping genes β-actin and GAPDH. All samples satisfied these criteria and were deemed suitable for Affymetrix GeneChip analysis (data not shown). Following hybridisation to the Affymetrix HG-U133A GeneChip, the scanned images were captured by the GeneChip Operating Software (GCOS) and converted to .DAT image files. The final data output from the GCOS in this experiment were Tab delimited text files for each of the ten chips in Microsoft Excel spreadsheet format. These spreadsheets contained numerical values for signal and present/absent/marginal calls for each of the probes.

The Hs578T(P) duplicate samples, Pa and Pb, were chosen as the comparison baseline and all samples were normalised to the mean of these samples using GeneSpring 6.0 software. Since samples were analysed in duplicate, advanced filtering was applied to the normalised data based on degree of fold change, with a cut off set at 2-fold increase or decrease. Additionally, the normalised data was filtered on flags and only those genes called present or

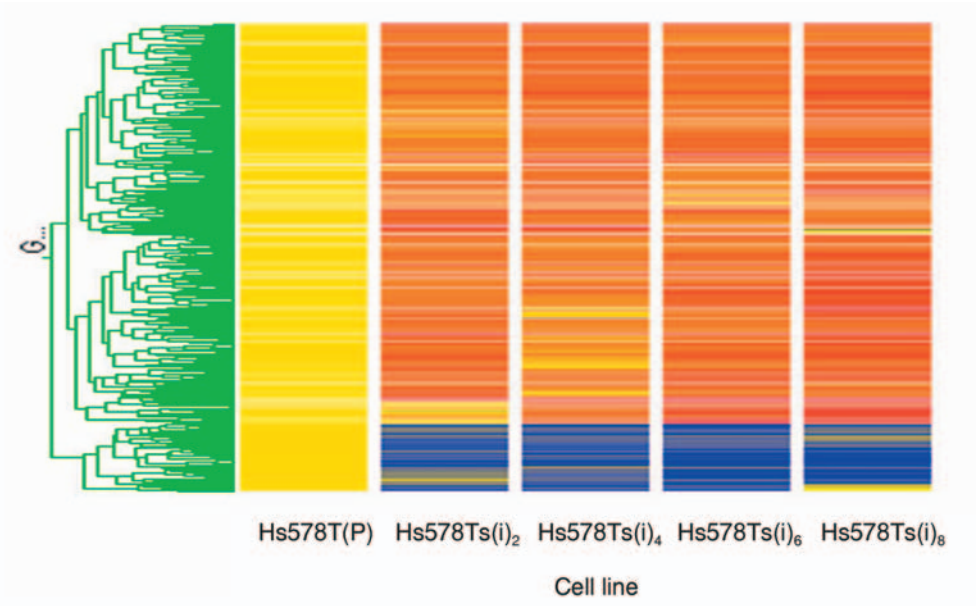

Figure 2. *GeneSpring 6.0 analysis identified 508 genes that were differentially expressed in the derivative cells relative to Hs578T(P). Data is represented as a gene tree, with the red colour representing up-regulated genes, blue representing down-regulated genes and yellow showing no difference in gene expression.*

marginal in two of the five conditions were allowed to pass the filter. This advanced filtering reduced the number of differentially expressed genes in the derivative cell lines relative to the parental cell line to 508 genes. These genes are displayed as a gene tree generated using cluster analysis (Figure 2). Out of the 508 differentially expressed genes, 71 (14%) of the genes were down-regulated and 437 (86%) of the genes were up-regulated.

Validation of microarray data using quantitative real-time RT-PCR analysis. In order to choose genes for further analysis, detailed literature surveys were conducted on the top 20 up- and down-regulated genes. From this analysis, 7 up-regulated (Table II) and 10 down-regulated (Table III) genes were chosen for validation in the Hs578T parental cell line and the most invasive subclone, $Hs578Ts(i)_8$. Duplicate reactions were conducted on each of 3 biological replicates, with data analysed using the delta delta CT method (10). Tables II and III show the fold increases as determined by DNA microarray and real-time quantitative RT-PCR analyses. When the log of the fold increase in gene expression as determined by real-time RT-PCR was plotted against the log of the fold increase as determined by DNA microarray analysis, there was excellent correlation between the two technologies (r^2=0.9249; Figure 3). MMP-2 was included in the validation even though it was below the 2-fold cut-off, as it had been previously shown to be differentially expressed in these cell lines.

Discussion

According to the World Health Organisation, cancer is currently the second biggest killer worldwide, with only heart disease killing more people each year. Breast cancer mortality, like many other cancers is related to metastatic spread of the primary tumour, with approximately 50% of all cancer patients showing evidence of metastasis at first presentation (2). In order for cells to metastasise, they must be capable of invading (11). Understanding the molecular mechanisms involved in tumour invasion and metastasis will lead to the development of new diagnostic and therapeutic agents.

DNA microarray analysis is a powerful tool in the armoury of the molecular biologist, as it allows examination of thousands of genes in parallel (12). However, vast quantities of data are generated requiring extensive data analysis and mining before final reliable results are obtained. The aim of this study was to identify invasion-specific or invasion-related genes using a series of increasingly invasive breast cancer cell lines. The series of sublines used in this study were established from the primary breast cancer cell line Hs578T using selection through an *in vitro* invasion assay system. Since these subclones were isogenic (*i.e.* share the same DNA), they provided a good model system to study invasion.

Cell lines were subjected to DNA microarray analysis using the Affymetrix HG-U133A array and data analysed using GeneSpring 6.0 software. The cut-off point chosen for

Table II. *Details of up-regulated genes validated with quantitative real-time RT-PCR.*

Gene title	Gene symbol	Function	Fold increase	
			Array	RT-PCR
Placenta specific 8	PLAC8	Role in placental enrichment and apoptosis	32	60
Cancer testis antigen 1B/NYESO1	CTAG1B	Tumour antigen	26	23
Cathepsin Z	CTZ	Cysteine-type peptidase activity	21	5
Keratin 18	KRT 18	Cell shape, size, embryogenesis and morphogenesis	7	12
Cytochrome b-245, alpha polypeptide	CYBA	Cytochrome b, oxidoreductase activity	6	16
Pirin	PIR	Transcription cofactor activity	4	8
Matrix metalloproteinase-2	MMP-2	Proteolytic enzyme	1.5	5

Table III. *Details of down-regulated gene validated with quantitative real-time RT-PCR.*

Gene title	Gene symbol	Function	Fold decrease	
			Array	RT-PCR
Insulin-like growth factor binding protein 5	IGFBP5	Interacts with insulin like growth factor	0.02	0.02
S100 calcium binding protein A4	S100A4	Calcium binding protein involved in regulation of cell cycle progression and differentiation	0.05	0.13
Decorin	DCN	Small leucine rich proteoglycan involved in matrix assembly	0.06	0.05
Sulfatase 1	Sulf1	Heparan sulphate proteoglycan involved in signalling	0.08	0.17
Calponin 1	CNN1	Actin and calmodulin binding	0.1	0.17
Lumican	LUM	Small leucine rich proteoglycan involved in collagen fibril organisation	0.14	0.08
Insulin-like growth factor binding protein 3	IGFBP3	Prolongs the half life of IGF	0.14	0.13
Serine proteinase inhibitor clade B member 2	SERPINB2	Plasminogen activator activity	0.17	0.45
V-yes-1 Yamaguchi sarcoma viral related	LYN	Receptor signalling protein tyrosine kinase activity	0.29	0.28
Tumour necrosis factor receptor superfamily member 11b	TNFRSF11B	TNF receptor	0.35	0.70

data analysis was 2-fold-up or -down change relative to expression of genes in the parental Hs578T cell line. A 2-fold cut-off is routinely used to identify genes that are most variable between samples. However, arbitrary 2-fold cut-offs in data analysis may exclude genes and, in particular, groups of genes from the same pathway whose differences, whilst less than 2-fold, have a significant effect when up- or down-regulated together (13).

Following GeneSpring 6.0 analysis, a list of 508 differentially regulated genes was generated; 71 (14%) of these genes were down-regulated and 437 (86%) were up-regulated. Following extensive literature surveys, 17 genes were chosen for further validation using quantitative real-time RT-PCR analysis. Placenta specific 8 (*PLAC8*) was the most up-regulated gene and insulin-like growth factor binding protein-5 (*IGFBP-5*) was the most down-regulated gene. The DNA microarray data and quantitative real-time RT-PCR data for these 17 genes showed excellent correlation.

The list of validated genes included several novel genes, such as PLAC8, not previously associated with invasion. *PLAC8* was originally identified by Galaviz-Hernandez *et al.* (14) using cDNA microarrays as a novel placental-enriched gene in mouse embryos. However, a recent paper identified *PLAC8* as onzin (a c-myc repressed target) and showed that onzin regulated the growth of the murine myeloid cell line, 32D (15). Onzin overexpressing cells showed loss of G2/M arrest and failed to up-regulate p53 in response to apoptotic stimuli. Several of the genes validated are known to play a role in tumour invasion and metastasis, such as *MMP-2* (16), *IGFBP-3* and *IGFBP-5* (17). Although some

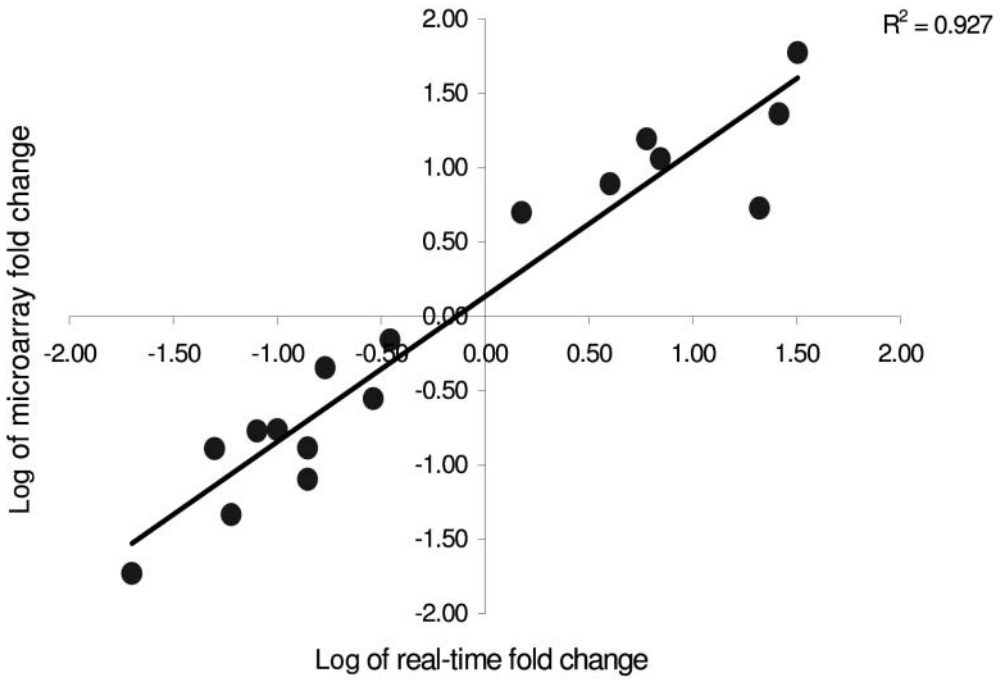

Figure 3. *Correlation of DNA microarray data and quantitative real-time RT-PCR data for 17 genes listed in Table II and III. Graph shows log of fold increase or decrease for array data and log of fold increase or decrease for quantitative real-time RT-PCR data.*

genes had no previous role in invasion, family members had been associated with invasion, *e.g.* Cathepsin Z (18). Other genes of interest included Lumican and Decorin that belong to a family of structurally related proteins with at least 13 members known as the small leucine-rich proteoglycans. There have been several reports in the literature showing that, when down-regulated together these proteoglycans are associated with poor prognosis for node-negative breast cancer (19).

The list of genes identified in this study were compared to results generated from three other published gene lists to see if any common genes could be identified. Van't Veer *et al.* (9) identified a 70-gene expression signature that was associated with poor prognosis. Ramaswamy *et al.* (20) published a list of genes associated with primary versus metastatic breast tumours. Sotiriou *et al.* (21) set out to identify genes associated with nuclear grade in breast cancer. When the 508 gene list was compared with the 70-gene poor prognosis gene signature, 5 common genes were seen (*CCNE2, COL4A2, IGFBP5, TSPY5* and *MELK*). Eight percent of the differentially regulated genes from this present study were common with the gene list generated by Sotiriou *et al.* (21) and 6% of the genes were common to the primary tumour gene list generated by Ramaswami *et al.* (20). There were no common genes in the 508 gene list and the breast metastasis list of 64 genes generated by Ramaswami *et al.* (20).

Conclusion

Using a series of isogenic, invasive subclones derived from the primary breast cancer cell line Hs578T, we have identified a panel of genes that may play a role in breast tumour cell invasion. Further experiments using gain- and loss-of-function studies will determine if any of these genes have potential as new therapeutic and/or diagnostic agents.

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