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Transcriptomic changes in human breast cancer progression as determined by serial analysis of gene expression

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Abstract

Introduction Genomic and transcriptomic alterations affecting key cellular processes such us cell proliferation, differentiation and genomic stability are considered crucial for the development and progression of cancer. Most invasive breast carcinomas are known to derive from precursor *in situ* lesions. It is proposed that major global expression abnormalities occur in the transition from normal to premalignant stages and further progression to invasive stages. Serial analysis of gene expression (SAGE) was employed to generate a comprehensive global gene expression profile of the major changes occurring during breast cancer malignant evolution.

Methods In the present study we combined various normal and tumor SAGE libraries available in the public domain with sets of breast cancer SAGE libraries recently generated and sequenced in our laboratory. A recently developed modified *t* test was used to detect the genes differentially expressed.

Results We accumulated a total of approximately 1.7 million breast tissue-specific SAGE tags and monitored the behavior of more than 25,157 genes during early breast carcinogenesis. We detected 52 transcripts commonly deregulated across the board when comparing normal tissue with ductal carcinoma *in situ*, and 149 transcripts when comparing ductal carcinoma *in situ* with invasive ductal carcinoma ($P < 0.01$).

Conclusion A major novelty of our study was the use of a statistical method that correctly accounts for the intra-SAGE and inter-SAGE library sources of variation. The most useful result of applying this modified *t* statistics beta binomial test is the identification of genes and gene families commonly deregulated across samples within each specific stage in the transition from normal to preinvasive and invasive stages of breast cancer development. Most of the gene expression abnormalities detected at the *in situ* stage were related to specific genes in charge of regulating the proper homeostasis between cell death and cell proliferation. The comparison of *in situ* lesions with fully invasive lesions, a much more heterogeneous group, clearly identified as the most importantly deregulated group of transcripts those encoding for various families of proteins in charge of extracellular matrix remodeling, invasion and cell motility functions.

Keywords: breast cancer, gene expression profiling, serial analysis of gene expression

Introduction

Invasive ductal breast carcinoma (IDC) is the most common malignancy of the breast, accounting for ~80% of all invasive breast tumors [1]. Although an issue of much controversy over the years, there is now general agreement and overwhelming histopathological and genetic evidence indicating that most invasive breast carcinomas appear to develop gradually from defined precursor lesions [2]. However, it also became clear that progression toward more aggressive phenotypes is not obligatory [3]. It is further evident that many genetic abnormalities underlying tumor progression are probably phenotypically silent.

Numerous molecular genetic changes have been reported as relevant in human breast carcinogenesis, such as anomalies affecting cell proliferation, apoptosis and invasion [4]. Preinvasive breast lesions such as high-grade ductal carcinoma *in situ* (DCIS) are known to have acquired a myriad

DCIS = ductal carcinoma *in situ*; IDC = invasive ductal carcinoma; NF = nuclear factor; SAGE = serial analysis of gene expression; TNF = tumor necrosis factor.

of genomic and transcriptomic changes, but as their name implies they are not yet invasive. The development of the ability to invade surrounding tissues is perhaps the most critical event in cancer progression. Among proposed invasion-related genes with reported altered expression in tumor cells are cell adhesion molecules, proteases and cytoskeletal molecules that may influence motility [5]. Identifying the key and most common gene expression abnormalities involved in the transition steps from preinvasion to a fully invasive phenotype is an extremely important topic of research and the main objective of the present report. Studies on this area may provide clues to better diagnose premalignant lesions at high risk of progression and may aid in achieving a better understanding of critical early molecular mechanisms involved in breast cancer evolution.

Serial analysis of gene expression (SAGE) is a comprehensive profiling method that allows for global, unbiased and quantitative characterization of transcriptomes [6,7]. SAGE provides a statistical description of the mRNA population present in a cell without prior selection of the genes to be studied, and this constitutes a major advantage. In this sense, only open systems can identify expressed genes that have not yet been cloned or partially sequenced. A second major advantage is that the information generated is digital in format, and can be directly compared with data generated from any other laboratory or with data available in public databases such as the Cancer Genome Anatomy Project http://cgap.nci.nih.gov/SAGE.

To perform a comparative SAGE analysis of normal, preinvasive and invasive lesions, we used a modified *t* test that we have recently developed [8]. This method has the advantage of taking into account both the intra-sample and inter-sample variability, identifying 'common patterns' of gene changes systematically occurring across samples. Most of the tests developed for measuring differential expression in SAGE data focus on capturing the first type of variation correctly, but tend to neglect the second type [9,10]. The aim of the present study was to provide a statistically robust global gene expression analysis on the progression of breast cancer using the described statistical approach comparing breast normal and tumor SAGE libraries obtained from public databases combined with additional SAGE libraries recently generated in our laboratory.

Materials and methods

SAGE libraries

To perform the comparative analysis of different stages of breast cancer progression, we combined SAGE libraries available in public databases with breast cancer libraries generated and sequenced at our own laboratory. To this end, 12 SAGE libraries of breast tissues (four normal breast tissues, six DCIS tissues and two IDC tissues) were downloaded from the Cancer Genome Anatomy Project – SAGE Genie database (libraries generated at the Polyak Laboratory, Dana-Farber Cancer Institute, Boston, MA, USA). We used 11 additional breast cancer SAGE libraries generated by ourselves, at an approximate resolution of 100,000 SAGE tags per library. All IDC SAGE libraries used in this study were from lymph node-negative, estrogen receptor-positive and progesterone receptor-positive tumor samples, with a tumor size classification of T1 or T2 (i.e. T1–T2 N0 M0). Table 1 summarizes all the SAGE libraries used in this comparative analysis.

SAGE methodology

For the SAGE libraries generated in our laboratory we followed standard methods. Briefly, total RNA was extracted from snap-frozen tissues using TRIzol (Invitrogen, San Francisco, CA, USA). SAGE library construction was performed with the I-SAGE kit (Invitrogen) according to the manufacturer's protocol and introducing only minor modifications. The anchoring enzyme was *Nla*III and the tagging enzyme used was *Bsm*FI. Concatemerized ditags were cloned into pZERO-1 and sequenced with an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

SAGE data processing

SAGE tags were extracted from sequencing files using the SAGE2000 software version 4.0 (a kind gift from Dr K. Kinzler, John Hopkins School of Medicine, Baltimore, MD, USA). Tag abundances for all libraries were normalized to a total of 100,000 tags (at which level a tag present 10 times has an abundance of 0.01%). Tag to gene assignments as well as additional annotations using public databases (e.g. Gene Ontology, Locus Link, Unigene cluster) were performed, using web-based SAGE library tools developed by ourselves http://spi.mdacc.tmc.edu/bitools/about/sage_lib_tool.html. In our comparison we used only tags with only one reliable assigned gene.

Statistical analysis of SAGE libraries

To compare the 23 SAGE libraries, we utilized a modified *t* test recently developed by us [8]. This analysis allowed us to identify SAGE tags with significantly different expression levels ($P < 0.01$) between normal tissue and DCIS and between DCIS and IDC. Tags with total counts of less than three in all libraries were filtered out before the analysis. In order to enable visualization and illustration of our analyses, we utilized the TIGR MultiExperiment Viewer (MeV 2.2) software (The Institute for Genomic Research, Rockville, MD, USA). This tool was employed for normalization and average clustering of the SAGE data.

The aim of the heat maps presented is simply to organize and illustrate the data by graphical means. Briefly, the normalization included logarithmic transformation followed by median centering by samples and genes. We used standard average hierarchical clustering techniques to classify

Table 1

Breast-specific serial analysis of gene expression (SAGE) libraries

Histology	Library name	Tag count	Unique tags
Normal breast tissue			
Normal 1	SAGE breast normal AP Br N[a]	37,419	15,886
Normal 2	SAGE breast normal epithelium AP 1[a]	49,021	18,276
Normal 3	SAGE breast normal organoid B[a]	58,326	19,602
Normal 4	SAGE breast normal organoid B2[a]	59,481	20,391
Ductal carcinoma *in situ*			
DCIS 1	SAGE breast carcinoma MD DCIS[a]	42,174	14,237
DCIS 2	SAGE breast carcinoma AP DCIS 3[a]	57,924	31,142
DCIS 3	SAGE breast carcinoma B DCIS 4[a]	60,699	20,224
DCIS 4	SAGE breast carcinoma B DCIS 5[a]	43,118	15,935
DCIS 5	SAGE breast carcinoma epithelium AP DCIS 6[a]	73,409	30,256
DCIS 6	SAGE breast carcinoma B BWHT18[a]	50,879	19,182
DCIS 7	MDACC 22T[b]	102,533	33,305
Invasive ductal carcinoma			
IDC 1	MDACC 09T[b]	91,647	37,863
IDC 2	MDACC 14T[b]	100,255	26,422
IDC 3	MDACC 15T[b]	90,198	27,653
IDC 4	MDACC 17T[b]	100,386	29,300
IDC 5	MDACC 18T[b]	101,543	29,936
IDC 6	MDACC 19T[b]	100,334	28,498
IDC 7	MDACC 20T[b]	100,047	28,903
IDC 8	MDACC 21T[b]	103,825	31,412
IDC 9	MDACC 24T[b]	99,546	30,363
IDC 10	MDACC 25T[b]	100,501	30,778
IDC 11	SAGE breast carcinoma B IDC 3[a]	68,937	22,732
IDC 12	SAGE breast carcinoma B IDC 5[a]	60,476	20,457
Total	23 breast libraries	1,752,678	

[a] Libraries available in public databases. [b] Libraries generated in our laboratory.

and illustrate further the differences found by the modified *t* test, showing the clusters of differentially coexpressed genes between the normal tissue, DCIS and IDC groups.

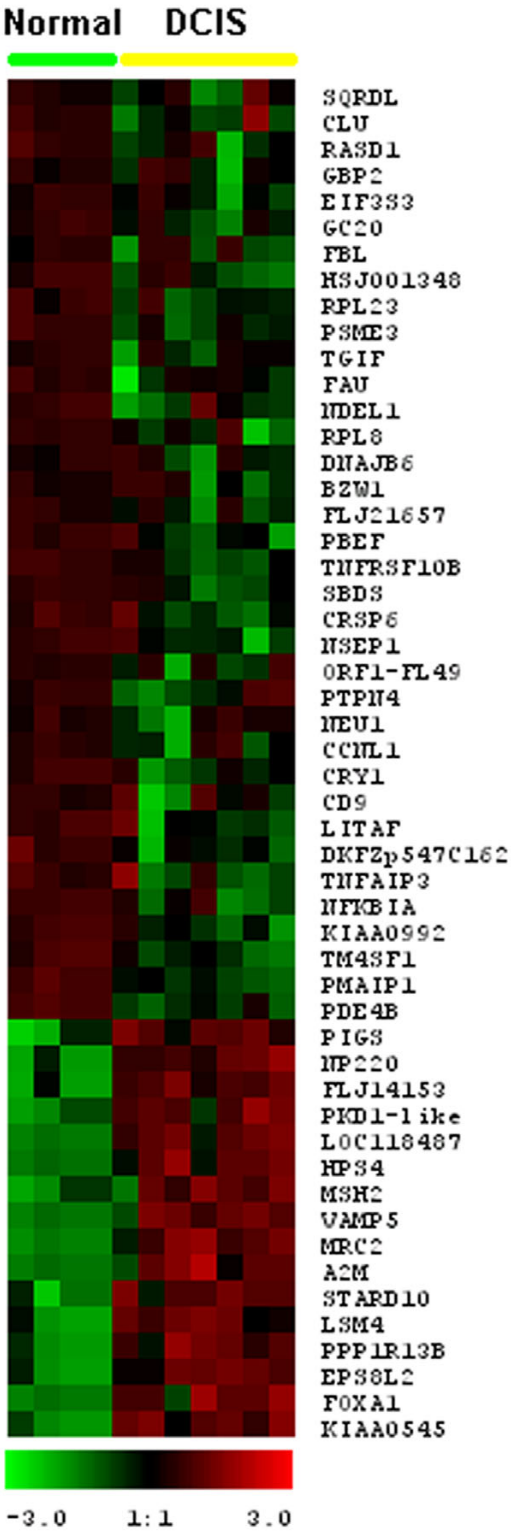
Results and discussion

Generation and analysis of SAGE libraries

The primary goal of our study was to identify the most commonly occurring transcriptome changes in the transition from normal breast epithelium to DCIS and invasive carcinoma. To this end, SAGE data obtained from 11 breast cancer libraries generated in our laboratory (1,090,815 tags) were combined and compared with data available in the public domain (661,863 tags), thus generating a dataset of almost 1.7 million breast cancer and normal specific tags, representing approximately 25,157 transcripts from a total of 23 libraries (Table 1).

Our statistically stringent analysis revealed 52 transcripts commonly deregulated across the board when comparing normal tissue with DCIS (Fig. 1), and 149 transcripts when comparing DCIS with IDC ($P < 0.01$) (Fig. 2) (see additional data files 1 and 2 for additional information with statistical cutoff at $P < 0.05$). Selected genes based on relative abundance, highly statistical differences and high fold changes between compared groups are sorted and represented in Tables 2 and 3.

Figure 1



Hierarchical clustering of the most commonly different expressed genes between normal breast tissue and ductal carcinoma *in situ* (DCIS) groups ($P < 0.01$). Color scale at bottom of picture is used to represent expression level: low expression is represented by green, and high expression is represented by red.

As expected, we detected various ribosomal genes among the most abundant transcripts in all the breast SAGE libraries, and these genes were highly upregulated in the invasive carcinomas. This agrees with the previous global expression profiles and with the comparisons of cancers and the corresponding normal tissues in general [7,11,12]. To simplify illustration of the data, ribosomal genes are not included in the figures and tables.

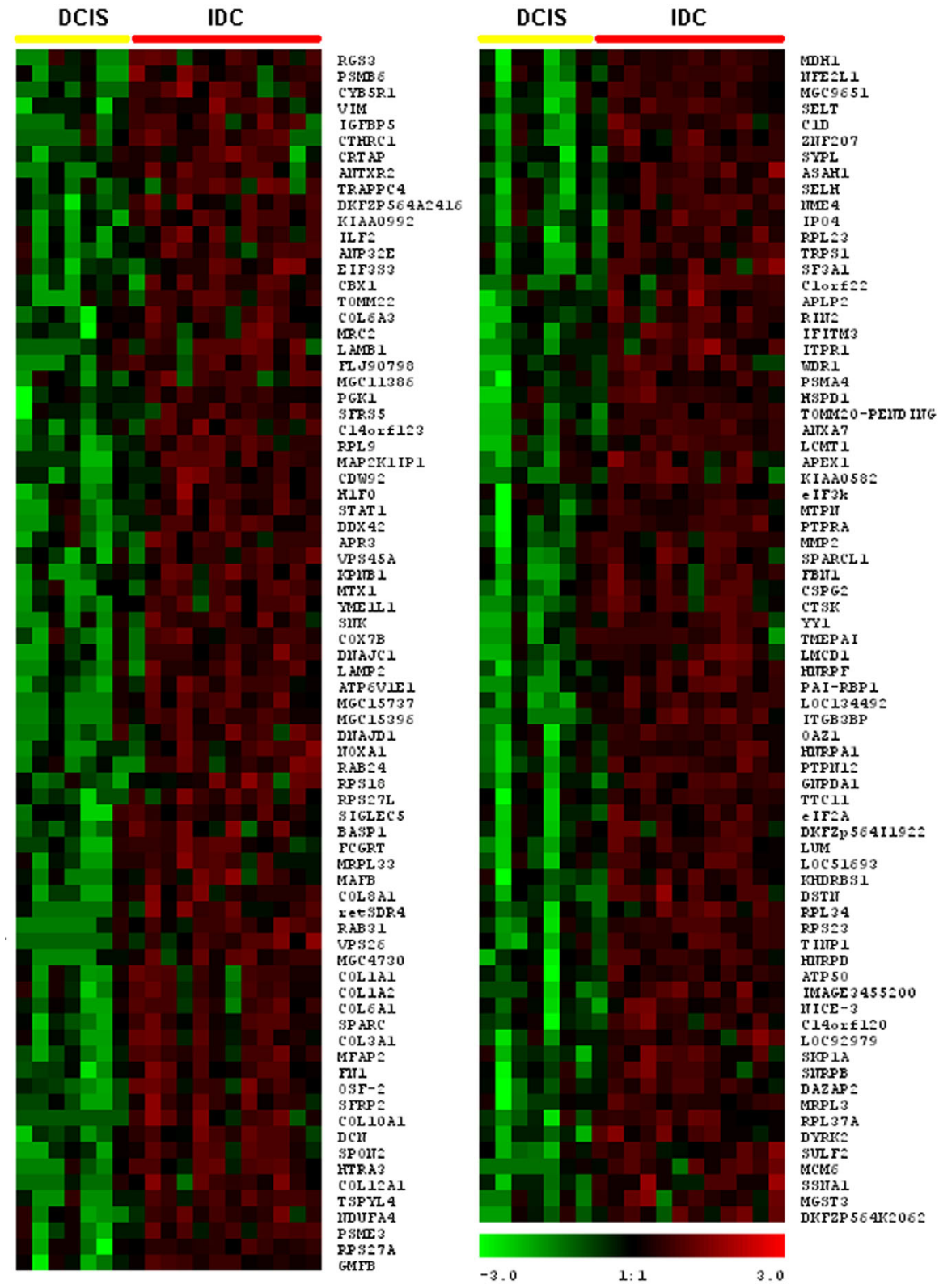
Global comparison of normal tissues and DCIS

Among the 52 transcripts detected as differentially expressed in DCIS ($P < 0.01$), 36 were downregulated transcripts and 16 were upregulated transcripts in these lesions when compared with normal breast epithelial cells and mammary epithelial organoids (Fig. 1 and Table 2). We defined and classified the 52 genes differentially expressed into the nine functional categories [13] shown in Fig. 3a. Interestingly, we found that 38% of these transcripts are related to the cell cycle (15%), signal transduction (8%) and apoptosis (15%).

As expected, our analysis of DCIS versus normal breast epithelium revealed numerous similarities with SAGE data reported previously [12,14], but more importantly it also provided novel information. The expression of numerous genes was significantly downregulated in DCIS, including: *transmembrane 4 super family member 1* (*TM4SF1*), *nuclear factor kappa light polypeptide* (*NFKB1A*), *pre-B-cell* (*PBEF*), *RAS dexamethasone-induced* (*RASD1*), *tumor necrosis factor receptor superfamily member 10b* (*TNFRSF10B*), and *tumor necrosis factor α-induced protein* (*TNFAIP*). All these transcripts were also observed downregulated in previous reports [12,14] (Table 2). On the contrary, our analysis revealed additional clusters of genes significantly downregulated in the DCIS group that were not previously reported by others: *clusterin/apolipoprotein J* (*CLU*), *nuclease sensitive element binding protein 1* (*NSEP1*), *lipopolysaccharide-induced TNF factor* (*LITAF/PIG7*), *basic leucine zipper/W2 domains 1* (*BZW1*), and *cyclin L1* (*CCNL1*) (Table 2).

Clusterin was one of the most dramatically downregulated genes (-63.9-fold; $P = 0.0036$) in DCIS libraries. This gene encodes a heterodimeric, highly conserved, secreted glycoprotein. Alterations in *Clusterin* expression and/or protein maturation are linked to changes in tissue growth or regression, which may be related to specific proapoptotic or antiapoptotic protein isoforms [15]. *Clusterin* was reported as overexpressed during tissue and cell involution, and was downregulated in esophageal squamous cell carcinoma and prostate carcinoma, suggesting that this expression alteration could be a general phenomenon during tumor progression [16,17]. On the contrary, and in contrast to these and our observations, Redondo and colleagues reported increased *Clusterin* expression in

Figure 2



Hierarchical clustering of the most commonly differentially expressed genes between ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) groups ($P < 0.01$). Color scale at bottom of picture is used to represent expression level: low expression is represented by green, and high expression is represented by red.

Table 2

Most frequent differentially expressed genes between normal breast epithelium and ductal carcinoma in situ (DCIS)

Tag	Gene	Description	Locus link	Fold change	*P* value
DCIS overexpressed genes					
GTATTTAACT	*PKD1-like*	*Polycystic kidney disease 1-like*	79932	13.7	0.0100
CGGACTCACT	*STARD10*	*START domain containing 10*	10809	11.2	0.0086
GTGTTGGGGG	*EPS8L2*	*EPS8-like 2*	64787	9.6	0.0099
TTTCTGGAGG	*KIAA0545*	*KIAA0545 protein*	23094	8.6	0.0100
GATAAATTAA	*FLJ14153*	*Hypothetical protein*	64747	8.5	0.0055
GAGAAATATC	*NP220*	*Nuclear protein*	27332	8.0	0.0088
CCCTCTTTGG	*LOC118487*	*mRNA similar to RIKEN cDNA 1110001019*	118487	7.4	0.0037
CTGGGACTGA	*LSM4*	*U6 small nuclear RNA associated* (S. cerevisiae)	25804	6.4	0.0055
CTGGGCCAGC	*VAMP5*	*Vesicle-associated membrane protein 5*	10791	6.4	0.0068
GCCCTTTCTC	*MRC2*	*Mannose receptor, C type 2*	9902	5.8	0.0015
TCTTGATTTA	*A2M*	*Alpha-2-macroglobulin*	2	5.8	0.0083
TAGTTTGTGG	*MSH2*	*MutS homolog 2, colon cancer*	4436	5.6	0.0091
TCAGTGAACT	*HPS4*	*Hermansky–Pudlak syndrome 4*	89781	5.6	0.0100
GTTTATTCTT	*FOXA1*	*Forkhead box A1*	3169	5.3	0.0044
GCCGCTGCCA	*PPP1R13B*	*Protein phosphatase 1*	23368	4.3	0.0054
TAAAGTGTCT	*PIGS*	*Phosphatidylinositol glycan, class S*	94005	3.9	0.0100
DCIS underexpressed genes					
GGGACGAGTG	*TM4SF1*	*Transmembrane 4 superfamily member 1*	4071	-442.6	0.0083
TAACAGCCAG	*NFKBIA*	*Nuclear factor kappa light polypeptide gene*	4792	-158.6	2.4×10^{-6}
CAACTAATTC	*CLU*	*Clusterin*	1191	-63.9	0.0036
GCCTTAACAA	*PBEF*	*Pre-B-cell colony-enhancing factor*	10135	-44.3	0.0020
GGGTTTTTAT	*NSEP1*	*Nuclease sensitive element binding protein 1*	4904	-36.2	0.0001
GACACGAACA	*RASD1*	*RAS, dexamethasone-induce 1*	51655	-31.4	0.0095
AAGATTGGTG	*CD9*	*CD9 antigen (p24)*	928	-29.6	0.0003
ACCAAATTAA	*TNFRSF10B*	*Tumor necrosis factor receptor superfamily*	8795	-29.4	0.0003
CTGGGCCTGA	*LITAF*	*Lipopolysaccharide-induced tumor necrosis factor*	9516	-28.9	0.0076
CTGCCATAAC	*SBDS*	*Shwachman–Bodian–Diamond syndrome*	51119	-24.2	0.0005
CACAGGCAAA	*BZW1*	*Basic leucine zipper and W2 domains 1*	9689	-22.1	0.0056
GTTCCCTGGC	*FAU*	*Finkel–Biskis–Reilly murine sarcoma virus*	2197	-22.1	0.0028
GTCTGCACCT	*DKFZp547C1*	*Hypothetical protein*	254851	-21.9	0.0087
TACGTTGCAG	*GC20*	*Translocation factor sui1 homolog*	10289	-21.8	0.0079
TGTAAAGATT	*CCNL1*	*Cyclin L1*	57018	-21.2	0.0008
TGTTAAGTTC	*CRY1*	*Cryptochrome 1 (photolyase-like)*	1407	-18.7	0.0091
GAAATAAAGT	*FLJ21657*	*Hypothetical protein*	64417	-18.5	0.0032
ATGGGCTTGA	*SQRDL*	*Sulfide quinone reductase-like (yeast)*	58472	-17.3	0.0061
TCAAGAAATT	*PSME3*	*Proteasome activator subunit 3*	10197	-15.7	0.0028
CCGTGGTCGT	*FBL*	*Fibrillarin*	2091	-15.3	0.0100

Table 2 *(Continued)*

Most frequent differentially expressed genes between normal breast epithelium and ductal carcinoma in situ (DCIS)

TGGAACAGGA	*TGIF*	*Transforming growth factor beta-induced factor (TALE family homeobox)*	7050	-12.2	0.0030
AATGCTGGCA	*DNAJB6*	*DnaJ homolog, subfamily B, member 6*	10049	-11.7	0.0065
AATGAGCAAC	*GBP2*	*Guanylate binding protein 2, interferon-inducible*	2634	-11.1	0.0082
GACCTATCTC	*KIAA0992*	*Paladin*	23022	-10.9	0.0092
AACTCTTGAA	*EIF3S3*	*Eukaryotic translation initiation factor 3*	8667	-10.6	0.0082
GGGATTTTGT	*PMAIP1*	*Phorbol-12-myristate-13-acetate-induced protein 1*	5366	-10.6	0.0091
AAAGCAAAAA	*PTPN4*	*Protein tyrosine phosphatase, non-receptor type 4*	5775	-10.4	0.0040
ACTGACTATC	*NEU1*	*Sialidase 1 (lysosomal sialidase)*	4758	-10.3	0.0095
TTCCAGTTCA	*PDE4B*	*Phosphodiesterase 4B, camp-specific*	5142	-9.9	0.0087
GAATGATTTC	*ORF1-FL49*	*Putative nuclear protein*	84418	-9.5	0.0070
GACTCGCTCC	*HSJ001348*	*cDNA for differentially expressed CO16 gene*	54742	-9.5	0.0072
TGGTTACAAA	*NDEL1*	*Nude nuclear distribution gene E homolog like 1*	81565	-8.9	0.0100
AGTATGAGGA	*TNFAIP3*	*Tumor necrosis factor, alpha-induced protein 3*	7128	-8.1	0.0083
CAGTTTAAAA	*CRSP6*	*Cofactor required for Sp1 transcriptional activation*	9440	-7.7	0.0100

breast carcinoma samples [18]. The reason for this discrepancy is unclear at this point. The role of *Clusterin* in cell survival, cell death and neoplastic transformation remains controversial [15].

Another commonly observed downregulated gene in DCIS libraries was *NSEP-1* (-36.2-fold; $P = 0.0001$). Also known as YB1, *NSEP-1* is a member of the highly conserved Y-box family of proteins, which regulate the transcription of several genes associated with cell death including both *fas*, a cell death-associated receptor, and the tumor suppressor gene p53 [19]. The decrease in expression of *NSEP-1* transcripts could play an important role in the early stages of breast carcinogenesis in order to overcome cell proliferation controls.

Interestingly, and as previously observed, we also detected significant downregulation of various cytokines and chemokines: *interleukin enhancer binding factor 2* (*ILF2*), *interleukin 13 receptor alpha 1* (*IL13RA1*), *leukemia inhibitory factor* (*LIF*), *cardiotrophin-like cytokine* (*CLC*), *chemokine C–C ligand 2* (*CCL2*), and *chemokine C–X–C ligand 1* (*CXCL1*). All these cytokines and chemokines are highly expressed in normal mammary epithelium and are dramatically downregulated in the DCIS samples. These differentially expressed genes were detected within a range of $0.02 < P < 0.05$ by means of the modified *t* test analysis. These small secretory molecules, although usually linked to inflammatory processes, could also play important autocrine and/or paracrine roles in the physiology of normal mammary epithelial cells in particular because receptors for these cytokines are also normally found expressed in normal breast epithelial cells [20]. Some of these molecules (e.g. *CXCL1*, *LIF*) appear to play important roles in the normal periodic cycles of growth and involution of the mammary gland following pregnancy and lactation. They may thus be part of the physiologic mechanisms associated with the massive apoptosis observed during involution [21,22]. Unfortunately we understand very little of the relevance of their intriguing *de facto* silencing in expression, both in *in situ* as well as in invasive breast cancer lesions.

Interestingly, we also detected a series of transcripts commonly overexpressed in the DCIS samples: *polycystic kidney disease 1-like* (*PKD1-like*), *START domain containing 10* (*STARD10*), *EPS8-like2* (*EPS8L2*), and *KIAA0545 protein* (Fig. 1d). One of these genes, *EPS8-like2*, encodes a protein that is related to epidermal growth factor receptor pathway substrate 8 (EPS8), and was shown to be essential in Ras/PI3K to Rac signaling [23]. *PKD1-like* encodes a member of the polycystin protein family. Members of this protein family may function in cell development and morphology, and may modulate intracellular calcium homoeostasis and other signal transduction pathways [24,25]. Although the *PKD1* gene has been associated with cancer mechanisms, this homologous family member has not been implicated in carcinogenesis processes to the best of our knowledge. KIAA0545, also known as *signal-induced proliferation-associated 1 like 3* (*SIPA1L3*), is a member of the *Sipa1* family and encodes a protein bearing a domain highly homologous to the catalytic region of human Rap1 GTPase-activating protein (Rap1GAP).

Table 3

Most frequent differentially expressed genes between ductal carcinoma in situ and invasive ductal carcinoma (IDC)

Tag	Gene	Description	Locus link	Fold change	*P* value
IDC overexpressed genes					
TGGAAATGAC	*COL1A1*	*Collagen type I, alpha 1*	1277	315.4	0.0054
ATGTGAAGAG	*SPARC*	*Secreted protein, cysteine-rich (osteonectin)*	6678	286.8	0.0003
TTTGGTTTTC	*COL1A2*	*Collagen type I, alpha 2*	1278	210.9	0.0084
TTGCTGACTT	*COL6A1*	*Collagen type VI, alpha 1*	1291	73.9	0.0023
TTATGTTTAA	*LUM*	*Lumican*	4060	56.7	0.0011
TTGGAGATCT	*NDUFA4*	*NADH dehydrogenase (ubiquinone)*	4697	56.4	0.0065
CCACAGGGGA	*COL3A1*	*Collagen type III, alpha 1*	1281	49.4	0.0056
ATCTTGTTAC	*FN1*	*Fibronectin 1*	2335	44.3	0.0031
TTGTAATCGT	*OAZ1*	*Ornithine decarboxylase antizyme 1*	4946	38.6	0.0038
TGTAATCAAT	*HNRPA1*	*Heterogeneous nuclear ribonucleoprotein A1*	3178	38.2	0.0039
GGAAGCTAAG	*OSF-2*	*Osteoblast specific factor 2 (fasciclin I-like)*	10631	36.3	0.0005
ACCTGTATCC	*IFITM3*	*Interferon induced transmembrane protein 3*	10410	34.3	0.0021
GGAAATGTCA	*MMP2*	*Matrix metalloproteinase 2*	4313	29.1	0.0008
TGCACTTCAA	*SPARCL1*	*SPARC-like 1 (mast9, hevin)*	8404	21.7	0.0050
GGAACTTTTA	*SULF2*	*Sulfatase 2*	55959	19.8	0.0017
CTGTTAGTGT	*MDH1*	*Malate dehydrogenase 1*	4190	18.5	0.0026
TATGAATGCT	*CSPG2*	*Chondroitin sulfate proteoglycan 2 (versican)*	1462	18.2	0.0017
TCCAAATCGA	*VIM*	*Vimentin*	7431	17.7	0.0014
TGTAGTTTGA	*SKP1A*	*S-phase kinase-associated protein 1A*	6500	16.9	0.0013
TAATAAACAG	*ASAH1*	*n-Acylsphimgosine amidohydrolase*	427	16.8	0.0085
GCCTCCTCCC	*EIF3k*	*Eukaryotic translation initiation factor 3*	27335	16.8	0.0098
GAAACAAGAT	*PGK1*	*Phosphoglycerate kinase 1*	5230	15.8	0.0006
TGCTTTGGGA	*TTC11*	*tetratricopeptide repeat domain 11*	51024	15.8	0.0018
GAAATCAAAA	*SIGLEC5*	*Sialic acid binding Ig-like lectin 5*	8778	14.7	0.0098
ATGTAGTAGT	*HNRPD*	*Heterogeneous nuclear ribonucleoprotein D*	3184	14.7	0.0025
GACCACCTTT	*MFAP2*	*Microfibrillar-associated protein 2*	4237	14.4	0.0000
ACTTATTATG	*DCN*	*Decorin*	1634	13.9	0.0007
TCTCTACCCA	*APLP2*	*Amyloid beta (A4) precursor-like protein 2*	334	13.7	0.0076
TGCAATATGC	*FBN1*	*Fibrillin 1*	2200	13.5	0.0037
ATTTCTTCAA	*SFRP2*	*Secreted frizzled-related protein 2*	6423	13.4	0.0030
ATAAAAAGAA	*CTSK*	*Cathepsin K (pycnodysostosis)*	1513	13.0	0.0003
GTACATTGTA	*MGC15737*	*Hypothetical protein*	85012	12.6	0.0011
TGATGTTTGA	*DAZAP2*	*DAZ associated protein 2*	9802	12.5	0.0013
TCCGTGGTTG	*BASP1*	*Membrane attached signal protein 1*	10409	12.1	0.0055
ACTGCTTTAC	*DKFZp564I1922*	*Adlican*	25878	12.0	0.0086
TCTGCAATGA	*TINP1*	*Trasnforming growth factor beta-inducible nuclear protein 1*	10412	12.0	0.0033
GTTTCTTCCC	*SELH*	*Selenoprotein H*	2880636	11.8	0.0037

Table 3 *(Continued)*

Most frequent differentially expressed genes between ductal carcinoma in situ and invasive ductal carcinoma (IDC)

AATATGCTTT	*ATP6V1E1*	*ATPase*	529	11.6	0.0009
TTATGGATCT	*SPON2*	*Spondin 2, extracellular matrix protein*	10417	11.3	0.0003
AAAATAAAGA	*APEX1*	*Nuclease, multifunctional DNA repair enzyme*	328	11.3	0.0099
TGTGTGTTTG	*HIF0*	*H1 histone family*	3005	11.2	0.0029
TATGTTTCAG	*PTPN12*	*Protein tyrosine phosphatase*	5782	11.1	0.0003
ACCAAAGCCC	*MGC9651*	*Hypothetical protein*	114932	10.6	0.0075
CAAGGATCTA	*NICE-3*	*NICE-3 protein*	25912	10.5	0.0057
GACGTCTTAA	*PSMA4*	*Proteasome subunit, alpha type*	5685	10.3	0.0038
CAGATAACAT	*TOMM20*	*Translocase*	9804	10.1	0.0064
AACTCTTGAA	*EIF3S3*	*Eukaryotic translation initiation factor 3*	8667	10.0	0.0047
TTCTTGGTGT	*TRPS1*	*Trichorhinophalangeal syndrome I*	7227	9.9	0.0042
TGCCTTAGTA	*DNAJC1*	*DNAJ homolog*	64215	9.8	0.0040
AGACAAGCTG	*SFRS5*	*Splicing factor*	6430	9.4	0.0015
ACAAGAATTG	*SYPL*	*Synaptophysin-like protein*	6856	9.3	0.0027
TACATCCGAA	*MTPN*	*Myotrophin*	136319	9.3	0.0013

Sipal1 is involved in the regulation of the Ras-mediated signal transduction pathway for cell proliferation and cell cycle progression [26]. These genes could be involved in signaling pathways that lead to cell proliferation, but their potential role in malignant transformation remains unknown.

Differentially expressed genes associated with NF-κB and tumor necrosis factor pathways

One of the transcripts observed to be most differentially expressed when comparing normal tissue with DCIS was *NFKBIA* (better known as IκBα), demonstrating a 150-fold higher expression ($P < 0.0001$) in normal mammary epithelial cells (Table 2 and Fig. 1b). *NFKBIA* is a member of IκB family genes that play a critical role in regulating the activity of the NF-κB transcription factor [27,28]. NF-κB plays a major role in diverse biological processes such as cell proliferation, differentiation, apoptosis and metastasis [29,30]. NF-κB is also required to prevent cell death induced by tumor necrosis factor (TNF) [31].

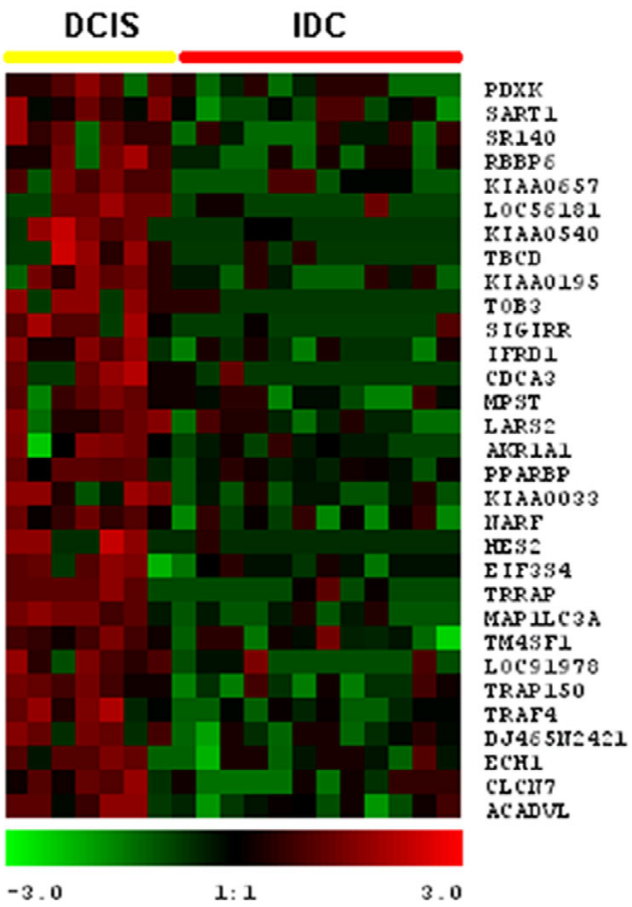
Interestingly, and perhaps pointing to connected pathways and related outcomes, we also detected a strong decrease in the expression levels of *TNFRSF10* (29-fold; $P < 0.0003$), *LITAF/PIG7* (29-fold; $P < 0.0003$) and *TNFAIP3* (eightfold; $P < 0.0083$) transcripts in the DCIS group. The protein encoded by *TNFRSF10*, also known as TRAIL/APO2, is a member of the TNF-receptor superfamily and contains an intracellular death domain. This receptor can be activated by TNF-related apoptosis inducing ligand and its role is to transduce apoptosis signals [32,33]. *LITAF/PIG7*, a transcription factor, termed lipopolysaccharide-induced TNF-alpha factor, also found downregulated, was reported to regulate TNF-alpha gene expression playing a major role in TNF-alpha activation [34]. This gene, also known as *P53-induced gene 7* (*PIG7*), has been shown to be induced by p53 when apoptosis is triggered, and therefore could also play a role in programmed cell death [35]. The concerted decline of these transcripts early in breast tumor progression appears conducive to a virtual silencing of apoptosis induction pathways and a consequential net increase in cell proliferation. In other words, the homeostasis of proliferation cell death normally operating in the breast epithelium is altered and inclined towards a net gain in cell numbers via multiple signaling pathways.

Global comparison of in situ and invasive carcinomas

We found 149 transcripts differentially expressed between DCIS and IDC at $P < 0.01$. All of these genes were found overexpressed commonly at the invasive stage (Fig. 2). Table 2 summarizes the 52 most commonly overexpressed genes in invasive carcinoma lesions. We defined and classified the 149 genes differentially expressed in 10 functional categories [13] as shown in Fig. 3b. Interestingly, we found that 37% of these upregulated transcripts are related to the cell cycle (12%), extracellular matrix or secreted proteins (13%), cell adhesion and motility (6%), and signal transduction (6%).

We were also able to detect 31 underexpressed genes in invasive carcinomas when compared with DCIS, but only when the stringency of the statistical comparison was dropped to within the 95% confidence interval (i.e. $P <$

Figure 3



Classification in functional categories of affected transcripts. **(a)** Differentially expressed between normal breast tissue and ductal carcinoma *in situ* (DCIS) ($P < 0.01$). **(b)** Transcripts differentially expressed between DCIS and invasive ductal carcinoma (IDC) ($P < 0.01$).

0.05), reflecting a lower level of consistency in these gene expression changes when comparing DCIS with IDC (Fig. 4). Examples of these genes include: *transmembrane 4 superfamily member 1* (*TM4SF1*) (-26.7-fold; $P = 0.04$), *tumor necrosis factor receptor-associated factor 4* (*TRAF4*) (-10.7-fold; $P = 0.04$), *PPAR binding protein* (*PPARBP*) (-8.2-fold; $P = 0.04$), *aldo-keto reductase family 1* (*AKR1A1*) (-6.7-fold; $P = 0.03$), *hypothetical protein dJ465N24.2.1* (-6.4-fold; $P = 0.028$), *microtubule-associated protein 1* (*MAP1LC3A*) (-3.7-fold; $P = 0.02$) and *retinoblastoma binding protein 6* (*RBBP6*) (-2.6-fold; $P = 0.04$).

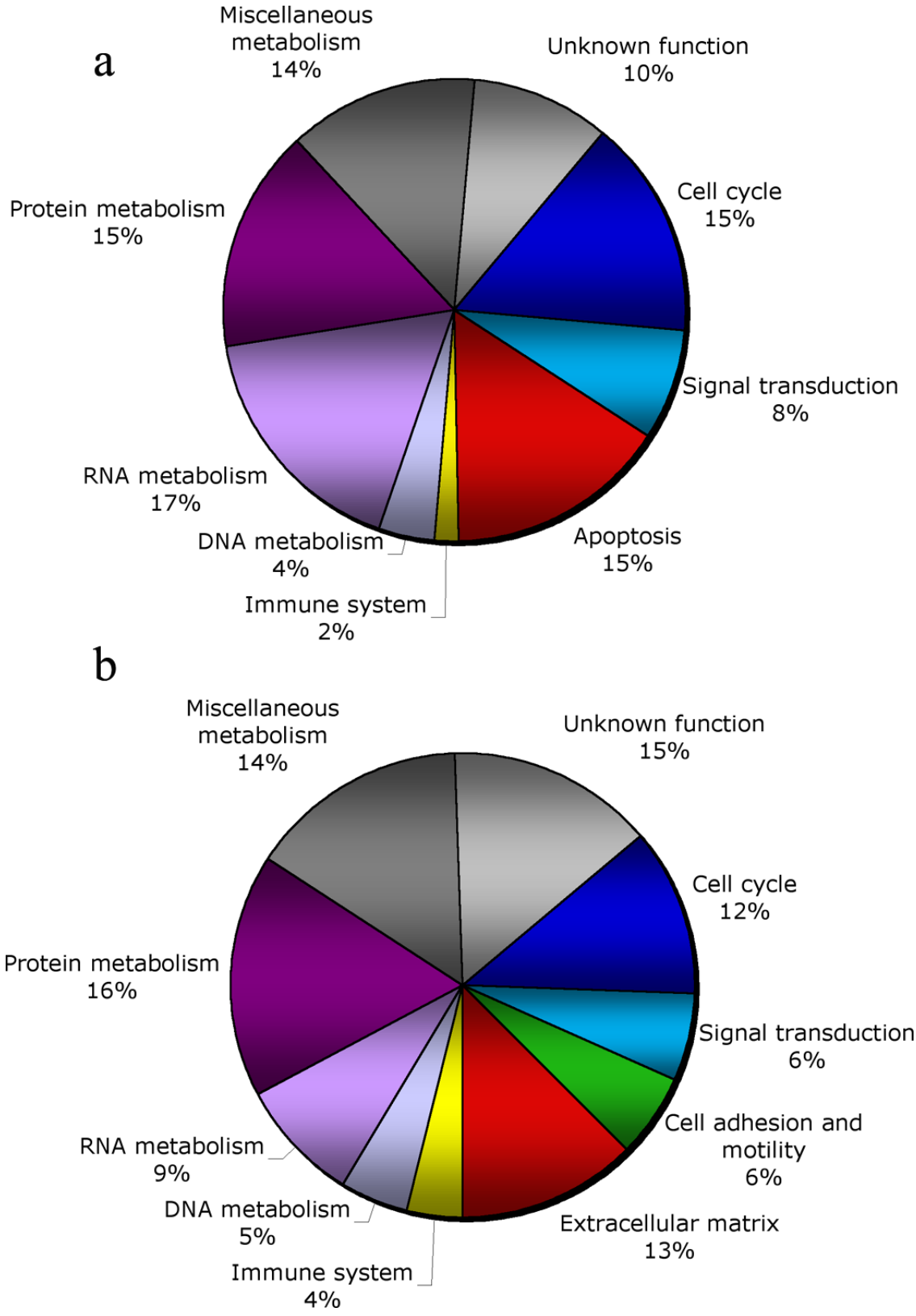
The first of these transcripts, *TM4SF1*, was also the most dramatically downregulated gene in DCIS when compared with normal breast libraries (-442.6-fold; $P = 0.0083$). The transmembrane proteins *TM4SF1*, also known as the *tetraspanin* superfamily, are implicated in diverse signal transduction events that play a role in the regulation of cell development, cell proliferation, differentiation and motility [36]. The *tetraspanins* are associated with adhesion receptors of the *integrin* family and regulate integrin-dependent cell migration [36]. In the present study, the loss in gene expression of *TM4SF1*, from normal breast tissue to invasive carcinomas, appears to be a common event in the progression of breast carcinomas. In addition, downregulated levels of the *TRAF4* transcript could cooperate in the evolution from DCIS to invasive carcinomas. *TRAF4* is a proapoptotic gene member of the TRAF family of adaptor proteins that mediate cellular signaling by binding to various members of the tumor necrosis family receptor superfamily and interleukin-1/Toll-like receptor superfamily [37]. Interestingly, a recent study showed that overexpression of *TRAF4* can induce apoptosis, playing a role in p53-mediated proapoptotic signaling in response to cellular stress [38].

Differentially expressed genes related with extracellular matrix remodeling and invasion processes

During their metastatic conversion, epithelial carcinoma cells acquire the ability to invade the surrounding tissues and later disseminate to secondary organs mostly via lymphatic vessels. The metastatic process is not just a function of acquisition of novel migratory and invasive properties by the epithelial tumor cells; the surrounding stroma also plays a critical role in this process [2]. Dramatic changes take place in order to remodel the extracellular matrix environment in response to the infiltrating cancer cells (desmoplastic reaction) [39-41]. In this sense, we identified high expression levels of several transcripts that could be a reflection of the host stromal response, such as *collagen 1α1*, *collagen 1α2*, *collagen 3α1*, *collagen 6α1*, *fibronectin I*, *fibrilli*, *microfibrillar-associated protein 2*, and *Spondin 2*.

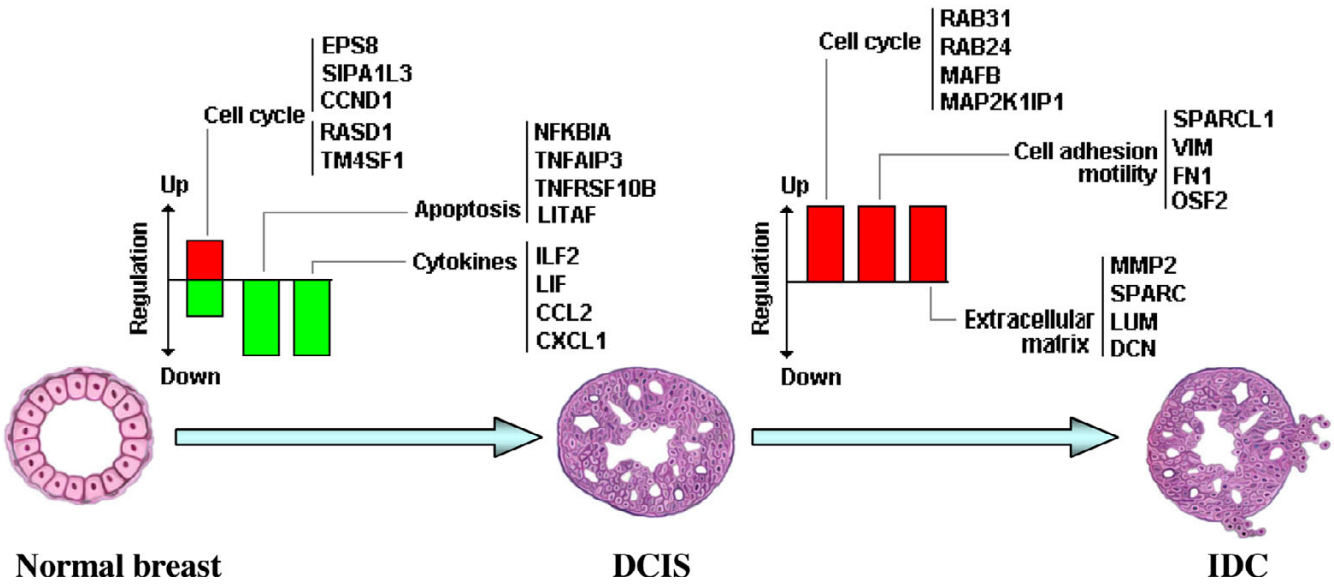
It is known that the proteolytic degradation of the extracellular matrix is more than the simple removal of a physical barrier to invasion; such processes and the increased expression of the involved genes are known to also significantly influence mechanisms controlling cell proliferation [42]. Matrix metalloproteinases are zinc-dependent endopeptidases involved in matrix degradation and tissue remodeling [43]. These endopeptidases are capable of degrading both the extracellular matrix and basement membrane, physical barriers that play important roles in preventing against expanding growth and migration of cancer cells [44]. It is therefore widely accepted that overexpression of matrix metalloproteinases is associated with cancer-cell invasion and metastasis. A member of the matrix metalloproteinase family (MMP-2) was highly expressed (29.1-fold; $P = 0.0008$) in IDC libraries in comparison with *in situ* carcinomas. MMP-2 has been shown overexpressed in various human tumors, including breast cancer [45,46].

Figure 4



Hierarchical clustering of downregulated genes in invasive ductal carcinoma (IDC) ($P < 0.05$). Color scale at bottom of picture is used to represent expression level: low expression is represented by green, and high expression is represented by red. DCIS, ductal carcinoma *in situ*.

Figure 5



Schematic model portraying some of the most significant transcriptomic changes observed in breast cancer progression. DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

To no surprise and as observed in other studies, we also detected significant increases in *SPARC* (286-fold; $P = 0.0003$) and a new related gene *SPARC-like1* (21.7-fold; $P = 0.005$) among the groups of genes upregulated in invasive lesions. The *SPARC* gene encodes for a secreted protein acid rich in cysteines also known as *osteonectin* [47]. This protein is involved in a variety of diverse biological processes including tissue remodeling, cell adhesion, proliferation, differentiation, matrix synthesis/turnover, angiogenesis and tumor cell migration and invasion [47]. Overexpression of the *SPARC* gene has been reported associated with melanoma and metastatic carcinomas of the breast, and increased *SPARC* expression has been observed in conjunction with increased *c-Jun* and *Fra-1* expression in a panel of invasive breast cancer cell lines [48].

Human *SPARC-like1*, also known as *mast9* or *hevin*, is a member of the SPARC protein family. Interestingly, previous reports indicated downregulation of *SPARC-like1* in prostate and colon carcinomas [49,50]. Contrary to these observations, we observed consistent high expression of this transcript across all IDC libraries. Functional assays suggest that *SPARC-like1* may serve as an antagonist to cell adhesion, playing a key role in the inhibition of attachment, and may facilitate spreading of endothelial cells on fibronectin substrates [51].

Taken together, these expression profiles suggest that *MMP-2*, *SPARC* and *SPARC-like1* are probably critical mediators of extracellular matrix remodeling and are all important in facilitating breast cancer invasion and progression.

Other genes commonly expressed at high levels in invasive carcinomas and of much lower expression in DCIS and normal breast tissues include *lumican* (*LUM/LDC*) (56.7-fold; $P = 0.0011$), *versican* (*CSPG2*) (18.2-fold; $P = 0.0017$), *vimentin* (*VIM*) (17.7-fold; $P = 0.0014$), *decorin* (*DCN/PG2*) (13.9-fold; $P = 0.0007$) and *adlican* (DKFZp564I1922) (12-fold; $P = 0.0086$). *Lumican* and *decorin* are members of the small leucine-rich proteoglycan family of proteins [40]. Several studies have demonstrated that small leucine-rich proteoglycan proteins can modulate cellular behavior, including cell migration and proliferation during tumor growth. Furthermore, the high expression level of *lumican* was associated with high tumor grade and was expressed specifically in breast cancer tissues, but not in normal breast tissues, suggesting that *lumican* is differentially expressed during breast tumor progression [40,52]. These findings suggest that *lumican* may play an important role in breast cancer growth.

Recent studies have suggested that expression of increased amounts of *versican*, a chondroitin sulphate proteoglycan, in neoplastic tissues may play a role in promoting tumor cell proliferation and migration [53]. Abnormal *versican* deposition has been observed in a number of tumor types, including breast cancer [54]. Furthermore, it has been suggested that the *versican*-rich extracellular matrices exert an anti-adhesive effect on cells, thus facilitating tumor-cell migration and invasion [55].

Vimentin is a type III intermediate filament normally expressed in cells of mesenchymal origin [56]. However, numerous studies have now demonstrated that *vimentin* can also be expressed in epithelial cells involved in physiological or pathological processes requiring epithelial cell migration [57]. *Vimentin* has indeed been described in migratory epithelial cells involved in embryological and organogenesis processes and tumor invasion [58]. Also, *vimentin* antisense transfection in vimentin-expressing breast cell lines was shown to reduce their *in vitro* invasiveness or migration, strongly emphasizing a functional contribution of *vimentin* to epithelial cell invasion/migration [59].

Conclusions

Using comprehensive gene expression profiling by means of SAGE combined with a recently developed statistical approach, we identified the most consistent and statistically significant changes occurring in breast cancer progression detected by this methodology. A comparison of the genes identified in our DCIS and IDC analysis with previous observations [11,12,14,41] revealed expected similarities. More importantly, several genes were identified in our analysis that were not previously reported or detected in other SAGE studies. This suggests that the comparative analysis we performed of normal breast tissue, DCIS and invasive carcinomas by means of the modified *t* test appears statistically rigorous and applicable to SAGE studies in which multiple libraries are compared.

In the present study we observed that deregulation of genes involved in the control of cell proliferation, apoptosis and mammary gland development are frequently altered at the *in situ* stage (Fig. 5). Meanwhile, alterations in the expression of genes related to the cell cycle and extracellular matrix remodeling (proteinases, collagenases, cysteine proteinases), and several transcripts related to cell adhesion and motility, were abundantly deregulated at the invasive carcinoma stage (Fig. 5). Additional analysis and validation of the identified genes will be required to determine the clinical value, and to determine whether they may constitute novel targets for translational research.

Competing interests

None declared.

Additional files

The following Additional files are available online:

Additional File 1

Complete list of differentially expressed genes between normal breast epithelium and ductal carcinoma in situ ($p < 0.05$). See http://breast-cancer-research.com/content/supplementary/bcr899-s1.xls
See http://www.biomedcentral.com/content/supplementary/bcr899-S1.xls

Additional File 2

Complete list of differentially expressed genes between ductal carcinoma in situ and invasive ductal carcinoma ($p < 0.05$). See http://breast-cancer-research.com/content/supplementary/bcr899-s2.xls
See http://www.biomedcentral.com/content/supplementary/bcr899-S2.xls

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