Gene expression profiles of the fibroblasts from breast tumors and normal tissue compared with the tumor expression profiles

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ABSTRACT

Objective: The study was designed to examine whether the gene expression profiles of fibroblast cell lines, established from the tumor and the normal tissue from the same breast, exhibit any similarities with the profiles of the original tissues.

Methods: Fibroblast cell lines were established from invasive ductal carcinoma (IDC) and ductal carcinoma in situ (DCIS) of the breast and the adjacent normal tissues. Isolated total RNA from the cell lines and tissues were used to prepare labeled cDNA which was hybridized to Becton Dickinson Atlas™ microarrays for obtaining profiles of expressed genes. The profiles of tumors and cell lines were compared. This study was carried out at King Faisal specialist Hospital and Research Center, Riyadh, Kingdom of Saudi Arabia, during 2004 and 2005.

Results: Alterations of expression of most of the genes in the tissues were not detectable in the cell lines. The expression of a lower number of genes was altered in DCIS compared with that in IDC tumors.

Conclusion: Although the fibroblasts discharge important functions, their gene expression profiles do not represent the breast tissue to the extent that any prognostic decisions could be made.

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The breast has a heterogeneous cellular constitution which varies with age and with the physiological state of the individual. In this environment, fibroblasts have important roles in breast homeostasis and cancer progression. Of particular relevance in this regard is the signaling through extracellular matrix between the stroma and the epithelium. This communication is instrumental in the differentiation of stromal cells into adipocytes,[1,2] and in the desmoplastic response to the tumor growth.[3] The tumor cell-fibroblast interactions are implicated in the invasion process, as the fibroblasts by up taking cathepsin D can cause the breakdown of extracellular matrix.[4] Cathepsin D is induced at elevated levels in the estrogen positive cells, while in the estrogen negative cells,[5] it is expressed contitutively.[6] Another pathway for the matrix breakdown involves the matrix metalloproteinase 2 (MMP-2) carried by the normal fibroblasts bound to collagen surrounding them. This MMP-2 is released by the cancer cells by the action of fibronectin on their surface.[7]

During breast cancer progression, some angiogenic factors, besides matrix degrading enzymes, are also produced by fibroblasts.[8-10] The

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expression of these macromolecules is regulated by NF-kB, a heterodimeric transcription factor. The expression of NF-kB, in turn, is regulated through the interaction between breast cancer cells and stromal fibroblasts. While the gene for the transcription factor NF-kB is an estrogen target, it is constitutively activated in the estrogen receptor negative tissue as a result of stimulation of 1kB degradation by IL-1 and TNFa. The NF-kB activation is accompanied by the induction of IL-6, promoting angiogenesis and urokinase plasminogen activator,[11] promoting matrix degradation. Cancer cell derived factors such as leukemia inhibitory factor, pleiotrophin, basal fibroblast growth factor, vascular endothelial growth factor, and epidermal growth factor have been implicated in the over expression of stromelysin III[12,13] insulin such as growth factors, gelatinase A, and hepatocyte growth factor in the stromal fibroblasts through the induction of activator protein 1, NF-kB, STAT, and serum response factors.[14,15] In view of this information, it is of considerable interest to examine the gene expression profiles of fibroblasts for a better understanding of the breast pathology.

In this study, we have looked into gene expression profiles in cells propagated from the tumors and the surrounding normal tissue under conditions promoting the growth of fibroblasts. The results suggest that the gene expression profiles of fibroblasts are considerably different from those of the tissues of origin.

Methods. ***Specimens.*** Normal and tumor tissues were obtained from 6 surgically resected specimens of diseased breasts of 6 patients, referred to King Faisal Specialist Hospital and Research Center, Riyadh, Kingdom of Saudi Arabia, during the year 2004. Informed consent of the patients was obtained, according to the institutional guidelines, for the use of surgical samples for research purposes. The specimens were immediately placed on ice and transported to pathology department to collect freshly cut normal and tumor tissues, which were then transported to the laboratory in culturing medium for the establishment of cell lines. Samples of tumors and adjacent normal tissues, for the study of gene expression, were immediately snap-frozen in liquid nitrogen, and stored at -80^{O}C until used. The clinicopathological profiles of the patients are detailed in the **Table 1**, giving age, grade, pre/post menopausal status (pre/post), estrogen receptor status (ER), progesterone receptor status (PR) and HER-2/Neu status.

Medium. Primary culture medium - Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen Corporation, USA) containing 20% Fetal bovine Serum (Hyclone). Sub-culture medium - RPMI 1640 (Invitrogen Corporation,USA) containing 1% Penicillin-Streptomycin (Gibco) and 20% fetal bovine serum (Hyclone).

Establishment of cell lines from tissues. Each specimen was minced using a sterile scalpel into very small pieces, which were placed in 60 x 15 mm cell culture dishes and covered with glass slips to enhance adherence. Culturing medium was added, and dishes were incubated in a humidified atmosphere at 37^{O}C with 5% CO_2. Dishes were observed daily, and medium was changed after every 3 days. Upon reaching confluence (25-30 days) cover slips were removed and tissues were discarded. Cells were rinsed with sterile phosphate buffered saline solution and subcultured. Cells were maintained onwards in sub culturing medium and trypsinized weekly, using Trypsin-EDTA. Almost all the cells had fibroblast morphology and microscopic examination revealed that more than 90% cells were fibroblasts. An immunocytochemical assay disclosed that the cell lines expressed the least levels of keratins. Epithelial cells express high levels of keratins. The cell lines, BCCL9, BCCL10, BCCL17, BCCL36 and BCCL37, were established from the tissues of patients BC25, BC26, BC33, BC36 and BC37. The ribonucleic acid (RNA) extraction, labeling and hybridization to Atlas human cDNA expression arrays. Total RNA was extracted from the cell lines and the pulverized frozen tissue samples with Trizol (Sigma, St. Louis, MO), followed by DNase I treatment to minimize any DNA contamination using message clean kit (GeneHunter Corp, Nashville, TN).

For this study, BD Atlas cDNA expression (Human Cancer 1.2) arrays were used. The Poly A+ RNA enrichment, cDNA labeling, hybridization and washing of the cDNA Atlas array membranes were carried out according to the instructions accompanying the arrays. Briefly, 40-50 μg of total RNA was used for Poly A+ RNA enrichment, which was used subsequently as template for cDNA probe synthesis, using α-^{32}P labeled deoxyadenosine triphosphate (dATP) (Amersham, UK). The labeled probes were purified by spin column centrifugation (Chroma Spin-200, Clontech laboratories). Hybridization of probes to the arrays were carried out at 68^{O}C in a rotation hybridization oven, using labeled probe (1x10^{6} cpm/1 ml of hybridization solution, BD Express Hyb). The membranes were then washed at 68^{O}C (4 times with 2x standard sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS), followed by 2 times 1x SSC, 0.1% SDS), and exposed for 5-7 days to phosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

Analysis of results was performed by developing phosphor Imager screen and comparing the images of normal and tumor samples and of fibroblast cell lines from these tissues by using Atlas image software (BD).

Significance of the difference between the ratios of signals in the cell lines, and tumors were evaluated by t test and *p* value of less than 0.05 was considered significant.

Results. The total RNAs extracted from the tumors, and the cell lines were used for preparing labeled cDNAs, which were hybridized to the BD Atlas human cancer arrays (with probes for 1176 genes). The gene expression profiles of tumors were compared with the profiles of the corresponding normal tissues and the ratios of tumors to normal were used for comparison. The profiles of 2 invasive ductal carcinoma (IDC) tumors and one ductal carcinoma in situ (DCIS) tumor have been presented for comparison. Except for BCCL17 which were propagated from the tissues of 65 years old patients, and BCCL37 (55 years old patients) the ages of the others are comparable (**Table 1**). The gene expression profiles of the cell lines, BCCL9, BCCL10 and BCCL17, were compared with those of IDC tumors and of BCCL36 and BCCL37 with those of DCIS and IDC tumors. The signals on microarray for a tumor and a cell line are presented in **Figures 1a and 1b**.

Transcripts of a total of 101 and 117 genes were detected in the tumors of patients of 39 (BC26) and 44 years (BC41). Only 34 transcripts were detected in the DCIS tumor (**Table 2**). Overexpression of 21 genes and underexpression of 10 genes were observed in both IDC tumors. **Table 2** represents the expression levels of genes detected in both tumors; the expression figures in this table are ratios of the intensities of signals for each gene between the tumor and the normal tissue or between the cell line derived from the tumor to the cell line derived from the corresponding normal tissue. Alteration of expression of 10 genes in common with the IDC tumors was detected in the DCIS tumor: 4 genes were underexpressed in contrast to IDC tumors in which case they were overexpressed; 5 genes were underexpressed as in IDC tumors, and only one gene, fibronectin precursor, was expressed at elevated level as in the IDC tumors (**Table 2**). Almost all the genes commonly affected in the 2 IDC tumors were unaffected in the cell lines, except for a few: adenosine diphosphate/adenosine triphosphate carrier protein gene was expressed at high level in 2 cell lines (one of IDC and the other of DCIS origin) as in the tumors (**Table 2**). Similarly, elevated expression of the high mobility group 1 protein and the vascular endothelial growth factor receptor was detected in 2 cell lines (both of IDC origin) as in the 2 IDC tumors. Notably, the expression of the cytokeratin 19 was detected at low level in the 3 cell lines, whereas it was detected at high levels in the tumors. Contrary to this, the c-jun proto-oncogene and cyclin-dependent kinase inhibitor 1 (p21) were found to be expressed at low levels in the tumors, whereas trends toward higher level expression were indicated in the cell lines. Three genes (cytokeratin 14, CYR61 protein and early growth response protein 1), expressed at low levels in all the 3 tumors (belonging to IDC and DCIS), were unaffected in the cell lines. It is notable that alterations in the expression of lowest number of genes were detected in the cell lines (BCCL 17 and BCCL37) established from the old patients with grade 2 tumors. Comparison between the 2 cell lines BCCL9 and BCCL10 propagated from ER- and ER + tumors of IDC type suggests differences in the levels of expression of ADP, HMG1, c-myc binding protein puff, cathepsin D precursor, Vascular endothelial growth factor receptor (VEGFR) and migration inhibitory factor (MIF), but due to the differences in the replicated experiments, the results are of preliminary nature.

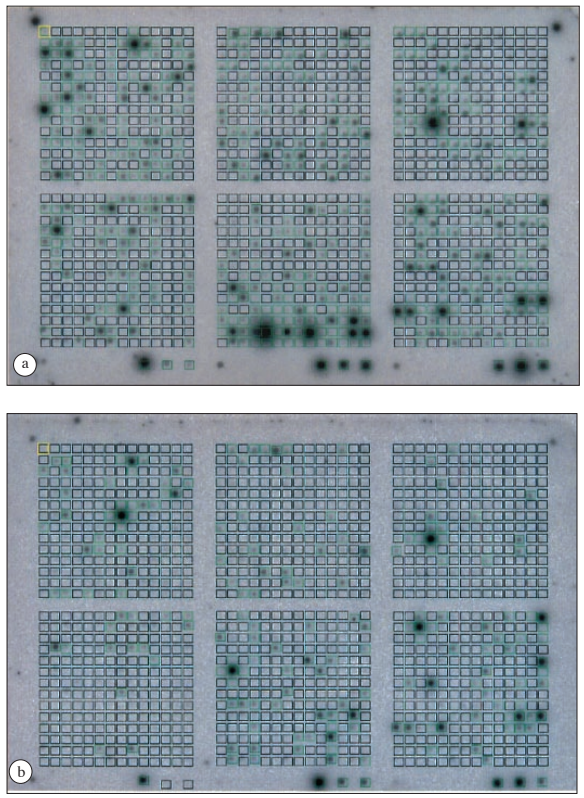

Figure 1 - Microarray grids with signals of expressed genes in a tumor and a cell line. The signals outside the frames are those of homeobox genes and of dots for positioning the frames. **a)** tumor (BC26), **b)** cell line.

Discussion. The breast is formed of epithelium and stroma. The stroma comprises, fibroblasts, pre-

Table 1 - Clinicopathological profiles of patients.

Parameters	Tumors			Cell lines				
	BC36	BC26	BC41	BCCL9 BC25	BCCL10 BC26	BCCL17 BC33	BCCL36 BC36	BCCL37 BC37
Type	DCIS	IDC	DCIS	IDC	IDC	IDC	DCIS	DCIS
Age (years)	36	39	44	47	39	65	36	55
Grade	3	3	3	3	3	2	3	2
Pre/Post	Pre	Pre	Pre	Pre	Pre	Post	Pre	Post
ER	Negative	Positive	Positive	Negative	Positive	Positive	Negative	Positive
PR	Negative	Positive	Positive	Negative	Positive	Negative	Negative	Positive
Her2/neu	Positive	Equivocal	Positive	Positive	Equivocal	Equivocal	Positive	Positive

At each column head, the cell line is mentioned by breast cancer cell line (BCCL) followed by a number, and the tissue from which it is derived is mentioned by **breast cancer** followed by a number. Pre/Post - pre or post menopause, ER - estrogen receptor, PR - progesterone receptor, DCIS - ductal carcinoma in situ, IDC - invasive ductal carcinoma

Table 2 - Gene expression profiles of tumors and cell lines

Gene/protein	Tumors			Cell lines							
	BC36 DCIS	BC26 IDC	BC41 IDC	BCCL9 BC25		BCCL10 BC26		BCCL17 BC33		BCCL36 BC36	BCCL37 BC37
				Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2		
ADP/ATP carrier protein	0.21	2.25	2.17	-	3.1	-	-	-	-	-	2.5
CENP-F kinetochore protein	-	2.57	2.2	-	-	-	-	-	-	2.15	-
Type I cytoskeletal 19 keratin	-	3.81	3.31	0.09	0.1	0.28	0.32	-	-	0.31	-
Hint protein; protein kinase inhibitor type I	0.48	3.49	4.63	-	-	-	-	-	-	0.4	-
Hepatoma derived growth factor	-	3.43	2.49	-	-	-	-	-	-	0.3	-
High mobility group 1	-	3.1	4.12		2.04	-	-	2.37	-	-	-
High mobility group protein 2	-	5.28	3.84	-	-	-	-	-	-	-	2.06
C-myc binding factor (puf)	-	3.12	2.97	-	-	-	0.5	-	-	-	-
Cathepsin D precursor	-	4.38	2.07	-	2.03	--	-	-	-	-	-
Fibronectin precursor	8.28	11.59	2.96	-	2.32	-	-	-		-	-
Vascular endothelial growth factor receptor	-	2.53	2.73	-	2.20	-	0.40	-	2.32	-	-
Protein kinase DYRK4	-	2.79	3.47	-	-	-	-	-	-	-	-
Nucleoside diphosphate kinase A	-	4.24	5.12	-	-	-	-	-	-	-	-
Calmodulin 1	-	2.31	3.06	-	-	-	-	-	-	-	-
Macrophage migration inhibitory factor	-	2.94	2.92	-	-	2.01	-	-	-	-	-
Ras-related C3 botulinum toxin substrate 1	0.42	2.12	2.57	-	-	-	-	-	-	-	-
Guanine nucleotide binding protein G(K)	-	2.75	2.08	-	-	-	-	-	-	-	-
Glutathione S-transferase	-	2.00	3.14	-	-	-	-	-	-	-	-
Desmoplakin I and II	-	2.27	2.03	-	-	-	-	-	-	-	-
Dual specificity A-kinase anchoring protein 1	0.34	2.17	4.93	-	-	-	-	-	-	-	-
Liver glyceraldehyde 3-phosphate ehydrogenase	-	2.14	2.58	-	-	-	-	-	-	-	-
Pleitrophin precursor	-	0.07	0.32	0.1	-	-	-	-	-	-	5.04
Cyclin dependent kinase inhibitor (p21)	0.45	0.16	0.21	4.91	2.45	2.20	0.4	-	-	-	-
Metalloproteinase inhibitor 3 precursor	-	0.41	0.23	0.42	0.15	0.11	0.3	-	2.98	-	-
C-jun proto-oncogene	0.16	0.27	0.21	2.03	-	2.12	-	2.64	-	-	-
Bone proteoglycan III precursor	-	0.36	0.43	0.3	2.47	-	-	-	-	-	-
Early growth response alpha	-	0.21	0.47	-	-	-	-	-	-	-	-
Cytokeratin 14	0.40	0.11	0.30	-	-	-	-	-	-	-	-
Collagen 6 alpha 3	-	0.43	0.21	-	-	-	-	-	-	3.36	-
CYR61 protein	0.48	0.41	0.46	-	-	-	-	-	-	-	-
Early growth response protein 1	0.26	0.01	0.61	-	-	-	-	-	-	-	-

Expression in each column is given as ratios of signal intensities between the tumor and the corresponding normal tissues, and as ratios of signal intensities between the cell lines propagated from the tumors and the corresponding cell lines propagated from the normal tissues.
ADP - adenosine diphosphate, ATP - adenosine triphosphate , CENP-F - centromere protein F, BC - breast cancer, DCIS - ductal carcinoma in situ, DYRK - dual specificity tyrosine (Y) phosphorylation regulated kinase 4, IDC - invasive ductal carcinoma, BCCL - Rep - repetition

adipocytes, adipocytes, endothelial cells, eosinophils and macrophages besides the extracellular matrix. The relative number of each cell type varies according to age, diet, hormonal status, and menopausal status. The identification of individual contribution of each cell type to the development of the malignancy and the invasive process has implications for improved treatment strategies. This study represents an attempt to examine the gene expression profile of the fibroblasts propagated from the tumors and the corresponding normal tissues. For this purpose, labeled cDNAs from the tissues and the cell lines were hybridized to cDNA arrays with probes for oncogenes, tumor suppressors, cell cycle control genes, genes involved in cell-cell interactions, signal transduction pathways, glycolytic metabolism, and apoptosis. The cell lines were not synchronized for this study as synchronization tempers with gene expression. Instead, the cells were cultured the same way and used at the same age of propagation (between passage 6 and 7).

Alteration of expression of more genes was detected in the IDC tumors compared with the DCIS tumor (**Table 2**). Only one gene, fibronectin precursor, transcript was elevated in all the 3 tumors. Fibronectin occurs, among other cells, on the surface of fibroblasts. It has roles in cell adhesion,[16] migration[17] and cytoskeletal organization and differentiation, wound healing and tumorigenesis.[18] Some of the overexpressed genes in both the tumors are known tumor markers: CENP-F gene product is involved in chromosomal segregation.[19] Keratin 19 protein is a constituent of the intermediate filaments.[20] It is synthesized in the epithelial cells to maintain structural integrity. High level expression of this protein has been associated with intermediate grade ER+ tumors[21] and with weakly invasive cell lines.[22] Hepatoma-derived growth factor is localized in the cytoplasm and its overexpression has been reported in normal tissues and tumor cell lines. It is mitogenic for fibroblasts.[23] High mobility group protein-1 (HMG-1) belongs to a group of chromatin associated nucleoproteins that facilitate the binding of various transcription factors to the pertinent DNA sequences.[24] It has been shown to enhance binding to DNA and transactivation functions[25] of p53. Transcriptional overexpression of HMG gene is highly correlated with malignant transformation and metastasis.[26] The HMG2 has been mapped to the translocation site in a variety of human mesenchymal tumors.[27] Its expression at high level has also been reported in a prostate cell line.[28] The enhanced expression of c-myc binding transcription factor puf suggests that c-myc expression is elevated in IDC tumors as in the case of prostate cancer.[28] Cathepsin D precursor, an aspartate proteinase, has roles in antigen processing and metastases and is a prognostic factor for breast cancer. Procathepsin D is mitogenic for breast cancer cells.[29] Vascular endothelial growth factor receptor promotes angiogenesis. The expression level of VEGFR, and its ratio to its ligand (VEGF) are prognostic indicators of primary breast cancer.[30] Desmoplakin forms part of the desmosomes and its expression is inversely correlated with tumor progression.[31] Its expression at high level has been reported in DCIS tumors.[21] Expression of glyceraldehyde 3-phosphate dehydrogenase, a key enzyme in intermediary metabolism, is correlated with histo-prognostic grading of breast cancer.[32] Migration inhibitory factor affects the anti-inflammatory and immunosuppressive activity of glucocorticoids. Its overexpression has been reported in primary breast cancer tissue.[33]

Expression of some interesting genes has been detected at low level in both IDC and DCIS tumors: cyclin dependent kinase inhibitor (p21), c-jun proto-oncogene, cytokeratin 14, CYR61, and early growth response protein 1 (**Table 2**). Low level expression of p21 indicates that mitotic check-point is not operating optimally while that of c-jun suggests that apoptosis is taking place at a minimum frequency. The CYR 61 protein promotes human fibroblast adhesion, migration and proliferation through the mediation of different integrins.[34] Early growth response-1 protein appears to be a pleiotropic gene with roles in transformation, proliferation, and chemoinvasion. Its decreased expression has been correlated with tumor formation.[35] Low level expression of c-jun, metalloproteinase inhibitor 3 and collagen VIα1 (instead of collagenVIα3 in our study) in weakly, and p21 in both weakly and highly invasive epithelial cell lines have been reported previously by Zajchowsky et al,[22] suggesting that epithelial cell lines may to a considerable extent represent the tumors.

Normal expression of approximately 15 of the genes, showing elevated expression in the tumors, has been observed in the breast cancer fibroblasts in our study (**Table 2**). Of the remaining genes, some are overexpressed in at least one cell line as in the tumors, whereas one (keratin 19) is underexpressed ($p<0.05$) in contrast to the elevated expression in the tumors. Two genes (metalloproteinase inhibitor 3 and bone proteoglycan III precursor) are underexpressed in 1-2 cell lines as in the tumor tissue. This observation suggests that the fibroblast cell lines are not similar to the tissues of origin, and do not reproduce the in vivo gene expression patterns of the tumors or the normal tissues. Our results are consistent with the previous studies using epithelial cell lines[21] showing limited reproducibility of the in vivo situation. Differences in

the gene expression profiles of tumors and epithelial cells have also been reported from studies applying sequential analysis of gene expression technique.[36] Contrary to the underexpression of p21 in epithelial cell lines in the study by Zajchowsky et al,[22] this gene is overexpressed in the 2 fibroblast cell lines and remains unaffected in the other cell lines in our study (**Table 2**), again suggesting that the malignant state is not represented by fibroblasts. The influence of the clinicopathological parameters, such as Her-2/neu, ER and PR status and grades are difficult to determine with this limited data, but a preliminary assessment suggests that a lower number of genes are altered in their expression in grade 2 compared with the grade 3 tumor cell lines. Since both the grade 2 patients are of old age (**Table 1**), the influence of this factor is not clear.

This study suggests that any identification of markers has to be based on the analysis of gene expression in the actual breast tumor. The conclusions based on the expression in the adjacent tissue or in the propagated cell lines need to be confirmed and reconfirmed.

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