Gene expression profiling in women with breast cancer in a Saudi population

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Objective: To generate consensus gene expression profiles of invasive breast tumors from a small cohort of Saudi females, and to explore the possibility that they may be broadly conserved between Caucasian and Middle Eastern populations.

ABSTRACT

الأهداف: توليد جوانب ظهور توافق الجين الجانبية لأورام الثدي الغازية من الدراسة الكتائبية للإناث السعوديات وذلك من أجل اكتشاف احتمالية حفظها بشكل عريض بين سكان القوقاز والشرق الأوسط.

الطريقة: أجريت هذه الدراسة كمستشار الملك فيصل التخصصي ومراكز الأبحاث بالرياض، بالنقلة العربية السعودية في الفترة ما بين يناير 2005م إلى يناير 2007م. تم توليد جوانب ظهور الجين من 38 ورم غازي للثدي و8 ورم من الأنسجة المجاورة (TATs) باستخدام (BD) منظمات ظاهرة (cDNA) تحتوي على 1176 جيناً. تم تأكيد النتائج بواسطة تفاعل سلسلة RT-PCR (الخمائر الناقلة القياسية) وتم تحليلها بواسطة طريقة فائقة السلسلة (RT—PCR) والبعد القياسي.

النتائج: تم التحليل 48 جين ظاهر وواضح في الأورام، ومنها تم الإبلاغ عن 25 بواسطة مختبرات الدراسات العربية. ظهر ثلاثة وأربعين (43) جينًا من هذه الجينات بشكل مختلف في TATs (الإنسدة المجاورة للورم). كان بالإمكان للبراءات أيضًا من التمييز بين الأورام و TATs بواسطة الجينات الأربعة الأعراض المائلة. كما يمكننا وضع البراءات في مجموعات وفقًا للتكنولوجيا للعلاج لدى المرضى بواسطة طريقة برهان البعد القياسي.

خاتمة: تشير نتائجنا إلى أن ظهور الجين الجانبية لدى المصابات السعودية بسرطان الثدي ومكان القوقاز يمكن ملاحظة إلى بعض مدى الرض، ويمكن استعمال ذلك في تصنيف المرضى وفقًا للمجموعات وتكهناتهم العلاقة، كما نقترح أيضًا ثلاث جينات ظاهرة مختارة (IGHG3, CDK6, RPS9) في الأورام والتي قد يكون لها دور روائي في سرطان النسيج، بالإضافة إلى دور TATs (الإنسدة المجاورة) والوحدة إلى اكتشافها وفقًا لذلك.

Methods: This study was performed at King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia, from January 2005 to January 2007. Gene expression profiles were generated from 38 invasive breast tumors, and 8 tumor adjacent tissues (TATs) using BD Atlas CDNA expression arrays containing 1176 genes. Results were confirmed by reverse transcriptase polymerase chain reaction, and analyzed by 2-dimensional unsupervised hierarchical clustering.

Results: The analysis identified 48 differentially expressed genes in tumors from which 25 are already reported by various western studies. Forty-three of these genes were also differentially expressed in TATs. The same data set has been able to distinguish between tumors and the TATs, interestingly by using only 4 of the differentially expressed genes. Moreover, we were able to group the patients according to prognosis to an extent by hierarchical clustering.

Conclusions: Our results indicate that expression profiles between Saudi females with breast cancer and the Caucasian population are conserved to some extent, and can be used to classify patients according to prognostic groups. We also suggest 3 differentially-expressed genes (IGHG3, CDK6, and RPS9) in tumors may have a novel role in breast cancer. In addition, the role of TATs is much more essential in breast cancer, and needs to be explored thoroughly.


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Breast cancer accounts for 21% of all female cancers in Saudi Arabia,\(^1\) and 22% in the world.\(^2\) It ranks at the top among cancers in females in Gulf Cooperating Council (GCC) countries. Due to the heterogeneous nature of this disease, breast cancer patients with similar prognostic types based on clinical and morphological criteria often show different therapeutic outcomes resulting in an inadequate or over-treatment of many patients. In recent years, gene expression profiling has been used in various human disorders including breast cancer, to expose molecular mechanisms responsible for the aggressive nature of the ailments with possible translational implementation. Analysis of variations in gene expression profiles of breast tumors by c-deoxyribonucleic acid (DNA) arrays have been successfully used for the classification of breast cancer into normal, baso epithelial, erbB2 over-expressing and luminal epithelial like groups (the last group was further divided into 2 subgroups with characteristic profiles and different prognosis). Several studies have identified gene expression profiles (or panel of genes) that correlate with complete or no response to chemotherapy of breast cancer.\(^3,4\) In other studies, elevated expression of genes involved in cell cycle, DNA replication, and chromosomal stability have been observed in groups of patients with poor prognosis of the disease.\(^5,6\) However, most of the gene expression profiling studies has been carried out mainly in the Caucasian population, and the inclusion of the non-Caucasian population is minimal. In view of the existence of genetic differences across regional or geographical locations, more data specific to indigenous populations are needed for effective comparative analysis of molecular changes in breast tumors among different populations. This study represents an effort in this direction, and is the first of its kind in the Saudi female population.

**Methods.** Specimen collection. We performed this study at King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia, from January 2005 to January 2007. Informed consent was obtained from each patient according to the Research Advisory Council (RAC) guidelines. Thirty-eight surgically resected primary breast tumor samples were obtained from patients (mean age 44 years, range 22-64 years) who received no prior chemotherapy, snap-frozen in liquid nitrogen, and stored at -80°C after macrodissection had been performed for pathological confirmation. For 8 different samples, tumor adjacent tissues (TATs), namely, the surrounding area of the tumor after being microscopically identified by the pathologist as non-tumor were also dissected, snap-frozen with liquid nitrogen, and stored at -80°C.

Histological assessment. Formalin-fixed, paraffin-embedded tissues were used to determine the tumor subtype (WHO classification), histological grade, lymphovascular invasion, margins status, number of involved lymph nodes, and the presence of extra-capsular invasion. Estrogen receptor (ER) status was determined by immunohistochemistry and measured as a percentage, and intensity of positive nuclear reactivities. For ERBB2 (HER receptor) immunohistochemistry, the Dako classification system (Herceptest) was used with scores of 0 and 1+ considered negative, and 2+ equivocal, and 3+ considered positive. A hematoxylin and eosin stained section was prepared before and after cutting slides for ribonucleic acid (RNA) isolation for assessment of the percentage of tumor cells. Only samples with greater than 50% tumor cells were selected, mean 67% and median 70% for all groups studied.

RNA extraction. Total RNA was extracted from the pulverized tissues with Trizol (Sigma), followed by DNase I treatment to minimize any DNA contamination using Message Clean kit (GenelHunter Corp, Nashville, TN) according to the manufacturer's instructions, and re-suspended in diethyl pyrocarbonate treated water. Quantification and purity of the RNA was assessed by calculating A260/A280 absorption ratio, and by electrophoresis in a denaturing formaldehyde/agarose ethidium bromide gel. Since corresponding control samples were not enough in most of the cases, Universal RNA, purchased from Clontech (Mountain View, CA), was used as control for those samples.

Gene expression arrays. For this study, gene expression profiles were generated by using cDNA Expression Arrays (BD Atlas Human Cancer 1.2) containing 1176 identified genes, which represent broad cellular functions. Further details of this array are available from the ClonTech website, [www.clontech.com](http://www.clontech.com). 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). Hybridization of labeled probes (1x10\(^6\) cpm/1ml of hybridization solution, BD Express Hyb) to the arrays (BD Atlas cDNA expression Arrays Human Cancer 1.2) were carried out at 68°C in a rotatory hybridization oven. The membrane was

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then washed at 68°C (4 times with 2 x SSC, 1% sodium dodecyl sulfate (SDS), followed by 2 times 1x SSC, 0.1% SDS, and exposed for 5 to 7 days to PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Analysis of results was performed after the development of PhosphorImager screen and global normalization of background to equalize the intensity of the signals on the membrane. The adjusted signal intensities of genes expressed in the tumor samples were compared with the adjusted signal intensities of genes expressed by control using Atlas Image software (BD). Genes were considered significantly differentially expressed in samples if they exhibited a 2.0 fold, or greater change in the adjusted signal intensities on comparison to control. The data were analyzed using hierarchical clustering software J-Express Pro 2.7.

**Reverse transcriptase polymerase chain reaction.** Total RNA (1 μg) was used to prepare cDNA using Moloney murine leukemia virus reverse transcriptase in a reaction mixture containing Tris-hydrogen chloride (HCl) 50 mM (PH 8), potassium chloride (KCl) 50 mM, magnesium chloride (MgCl₂) 6mM, dithiothreitol (DTT) 5mM, oligo (dT)₁₅ 50 pmol. The reaction mixture was incubated at 42°C for one hour. The same volume of cDNA mixture along with the respective primers at 50 pmol concentration were used for reverse transcriptase-polymerase chain reaction (RT-PCR) of the selected genes. The thermalycler was programmed as follows with the range of Tₘ's from 58-62°C: initial denaturation at 94°C for 2 minutes followed by 26-28 cycles of denaturation at 94°C for one minute, annealing at 58°C for one minute, extension at 72°C for one minute, and final extension at 72°C for 5 minutes. The PCR products were resolved electrophoretically on a 1% agarose gel stained with ethidium bromide and evaluated visually, and by using densitometry. Eight genes were evaluated for validation of the results from array data.

**Results.** We identified a sub-set of 48 genes that were significantly differentially expressed in at least 9 out of 38 (25%) tumor samples in comparison to control. In this set, 22 genes were found to be over-expressed, and 26 genes under expressed. However, one gene VEGF1, was found to be over-expressed in only 6 tumors. Interestingly, this gene was one of those few genes for which differential expression was not significant in the TATs. A total of 43 genes from the above data set was found to be differentially expressed in TATs, 18 genes were over expressed and 25 genes were under-expressed in comparison with control. Genes were considered significantly over, or under expressed if they show at least 2-fold change in expression in comparison with control, and the changes were statistically significant (p<0.05). Some of the over-expressed genes are known to be involved in cell matrix interactions, cell shape, suppression of host defense, metastases, and synthesis. Among these genes are fibronectin, keratin 7, interferon induced transmembrane protein 9-27, purine-binding transcription factor (Puf) and fatty acid synthase. The under expressed genes include; ubiquitin C, tubulin alpha, cadherin 5, gluthathione S-transferase, Tumour necrosis factor type 1 receptor associated protein and Elongation factors 1 and 2 are known to be implicated in protein degradation, maintenance of cell shape, adhesion, protection from oxidative damage, apoptosis, and protein synthesis. Interestingly, we observed 5 genes that are significantly differentially expressed in tumor samples and not in TATs when compared with universal RNA. These are Immunoglobulin heavy constant gamma 3 (IGHG3), Cadherin associated protein (α-catenin), Cyclin-dependent kinase 6 (Cdk6), Vascular endothelial growth factor Receptor-1 (VEGFR1) and ribosomal protein S9 (RPS9). Shown in Table 1 is a list of some known functions of these genes and their possible links with tumorigenesis. Unsupervised hierarchical clustering using 4 of these genes successfully separates the 8 TATs and corresponding tumor samples (Figure 1). Quantitative RT-PCR was performed on some selected genes among the 48 signature gene set including; fibronectin (FN1), c-MYC purine-binding transcription factor (Puf), vascular endothelial growth factor receptor 1 (VEGF1), keratin 7, fatty acid synthase.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Implication in cancer</th>
<th>N = 38 n (%)</th>
</tr>
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<tbody>
<tr>
<td>IGHG3</td>
<td>Immunoglobulin isotype</td>
<td>Squamous NSCLC&lt;sup&gt;44&lt;/sup&gt; Ulcerative colitis&lt;sup&gt;35&lt;/sup&gt; Hairy Cell Leukemia&lt;sup&gt;36&lt;/sup&gt;</td>
<td>19 (50)</td>
</tr>
<tr>
<td>Cdk6</td>
<td>Proliferation and differentiation of multiple cell types</td>
<td>Non-Hodgkin B lymphoma&lt;sup&gt;28&lt;/sup&gt;</td>
<td>14 (37)</td>
</tr>
<tr>
<td>Catenin</td>
<td>Cell-cell adhensive cadherin-catenin complex</td>
<td>Breast cancer&lt;sup&gt;37&lt;/sup&gt; Ovarian cancer&lt;sup&gt;79&lt;/sup&gt; Endometrial carcinoma&lt;sup&gt;39&lt;/sup&gt; Colon cancer&lt;sup&gt;40&lt;/sup&gt;</td>
<td>13 (34)</td>
</tr>
<tr>
<td>RPS9</td>
<td>A ribosomal protein subunit</td>
<td>Pancreatic adenocarcinoma&lt;sup&gt;29&lt;/sup&gt; Glioblastoma multiforme&lt;sup&gt;50&lt;/sup&gt;</td>
<td>13 (34)</td>
</tr>
<tr>
<td>VEGF1</td>
<td>A vascular endothelial growth factor</td>
<td>Breast cancer&lt;sup&gt;41&lt;/sup&gt; ALL&lt;sup&gt;20&lt;/sup&gt;</td>
<td>6 (16)</td>
</tr>
</tbody>
</table>

* Tats - tumor adjacent tissues, RNA - ribonucleic acid, IGHG - immunoglobulin heavy constant gamma, Cdk - cyclin-dependent kinase, RPS - ribosomal protein, VEGF - vascular endothelial growth factor, NSCLC - non-small cell lung cancer, ALL - acute lymphoblastic leukemia
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Figure 1 - Unsupervised hierarchical clustering of the 8 tumor (black) and 8 corresponding TATs (red) samples using only 4 of the genes that are significantly expressed in tumor and not in TATs in comparison to universal ribonucleic acid. Each column refers to a patient and each row to a gene. Red indicates a high value of gene expression and green indicates low value. Genes with median expression are in black.

Figure 2 - Reverse transcriptase-polymerase chain reaction analysis confirming the expression profile for some of the genes obtained from the microarray gene filter. All genes were significantly up-regulated in tumor (T), in comparison to samples containing basal levels of the gene (N). β-actin was used as a loading control in all cases. Densitometry was also used to confirm the observed differences between samples (data not shown).

Figure 3 - Unsupervised hierarchical clustering of the 8 tumor (black) and 8 corresponding TATs (pink) using the 48-gene set. Each column refers to a patient and each row to a gene, listed. Red indicates a high value of a gene expression and green indicates a low value. Genes with median expression are black.
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synthase (FAS), vimentin (VIM), and (CTNNA1) and immunoglobulin heavy constant gamma 3 (IGHG3). There was a good correlation between the RT-PCR data and gene expression results as shown in Figure 2. Further confirmation was achieved using densitometry (data not shown). This confirms our observation that these genes indeed have differential expressions in breast tumors. The 48-gene set was used to group the expression profiles of samples on the basis of their overall similarity using the unsupervised hierarchical clustering. In the resulting dendrogram, it was possible to observe grouping of tumors separately from the TATs (Figure 3). We also examined the possibility to predict disease outcome based on the differentially expressed 48-gene set using clustering analysis. Follow-up data were collected 24-36 months after initial diagnosis of patients. At the time of study, out of 38 patients, 23 were still in remission, 7 were free from disease for 6 months and then showed evidence of local recurrence or metastasis, 6 were never free from disease, and 2 did not appear for follow up. The patients were categorized into 3 groups, patients who never had disease free survival, patients with recurrence or metastasis, and patients in remission. We observed tendency of clustering to an extent, however, there was no clear pattern of patient outcome (data not shown).

Discussion. The incidence of breast cancer has been associated with some common risk factors in patients across different ethnicity and geographical locations. However, despite some similarities in the risk factors, clinical differences resulting in different therapeutic outcomes have been reported among patient cohorts in different parts of the world, implying the existence of genetic differences across regional or geographical locations. Recognized breast cancer risk factors in the West such as nulliparity, low parity, late age of first pregnancy, never having lactated, are all a strong part of Saudi society, yet breast cancer incidence is still high among women from the Kingdom of Saudi Arabia (KSA), which highlights the need for study of underlying genetic mechanisms.

Several Western investigators have used cDNA expression arrays containing thousands of gene sequences or differential cloning methods to generate gene expression profiles for breast cancer, to explore the heterogeneous nature of this disease, however, these studies lack data from middle eastern populations. In this study, gene expression profiles from Saudi females with invasive breast cancer were generated, and compared with the already-identified panel of genes in Caucasian population. The array in this study consisted of only 1176 probes, however, it represents genes that are frequently implicated in different stages of neoplastic transformation. Therefore, our findings are well comparable with other studies in which several thousands (approximately 30,000) gene probes have been used. The 48-gene set identified by this study contains more than 50% (25/48) of the genes already reported in several Western studies. Thus, suggesting similarities in gene profiles between Western and Middle Eastern populations. Twelve of the genes identified in our study genes have been correlated with breast cancer progression including HMGA1, PUF, Fibronectin, Ubiquitin C, IFITM1, Desmin, RhoB, Vimentin, and Keratin 7. This further suggests that the remaining genes observed in this study may also aid in better understanding of the molecular events involved in the cellular development of this disease.

Perhaps the most interesting finding in the current study was the identification of 5 genes that were significantly differentially expressed in tumor tissue and not in TATs in comparison with universal RNA. Two of these genes, α-catenin and VEGF1, have already been implicated in breast tumor. The α-catenin gene may also be important as a prognostic marker as alterations in the E-cadherin-catenin complex have been suggested as being of prognostic value in breast cancer. The T1 breast tumors (Tumor stage 1) harbor less alterations in E-cadherin-catenin complexes and are less likely to spread making the prognosis better for these patients in comparison with those diagnosed with increased alterations detected in tumors at T2 (Tumor stage 2). Therefore, the entire cadherin-catenin complex should be evaluated to assess its prognostic value in breast cancer. The VEGF1, like α-catenin is essential for breast cancer progression, and has also been shown to be important in the organ-specific tumor spread. The expression level of VEGF1 and its ratio to its ligand (VEGF) are prognostic indicators of primary breast cancer. It was interesting that it is not detected to be differentially expressed in any of the TATs in our study, which may suggest that it has a novel role restricted to breast tumor cells only, and VEGF, which is frequently located in the stroma is needed to control the tumor microenvironment. Although no significant role for IGHG3 has been identified in breast cancer yet, it has been detected to be differentially expressed in other gene profile studies for breast cancer. In our study, the gene was not identified in TATs, however, 19 tumor cases have shown differential expression of IGHG3. Similarly, the deregulated expression of Cdk6 (and its partner, cyclin D3) has already been shown to predispose cells to malignant transformation, and is implicated in the onset of non-Hodgkin B lymphoma. The RPS9 is the only under expressed gene to be identified in tumors and not in TATs. It has never been implicated in breast cancer tumorigenesis, however, it may have an important role.
as it has already been identified in pancreatic cancer and malignant astrocytic gliomas. As it is down regulated in these tumors in comparison with normal tissues, it has been implicated as an important genetic marker in these diseases. Therefore, we suggest the exciting potential that these 3 genes, JGHC3, CDK6, and RPS9, may also be important for the progression of the disease. Indeed, the ability of 4 of these genes to group tumor samples separately from TATs by hierarchical clustering (Figure 1) further suggests that these genes may also have potential as possible markers for breast cancer. We also suggest that the TATs may provide the base from which the recurrence of the tumor occurs as their gene profile is very similar to the tumors. Thus, they may have an important role in the tumorigenesis of the disease, like stroma. The microenvironment created by the breast stroma compartment is currently identified as playing a pivotal role in carcinogenesis and represents a valid target in breast cancer therapy. Breast tumors contain ‘reactive stroma’ that is characterized by modified extracellular matrix (ECM) composition, increased microvessel density, inflammatory cells, and fibroblasts with an ‘activated’ phenotype.

The 48-gene data set was also used to construct a dendrogram based on unsupervised hierarchical clustering analysis in order to group patients according to prognosis. We observed slight clustering, whereby patients never free from disease have a tendency to cluster together. However, due to the limited power of the unsupervised technique of hierarchical clustering, or due to limited sample size used in this study, it was difficult to predict accurate prognosis.

We conclude that analysis of a much larger cohort of patients with similar disease subtypes, and a sufficient follow-up period is necessary to generate a molecular taxonomy of breast cancer and to determine the efficiency of clustering analysis as a prognostic tool. Moreover, studying the genes from the data set identified by our study may provide a promising area for future research efforts, contributing valuable information on the molecular biology of the disease, and possible therapeutic relevance.

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References

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