Identification of *ApoA1*, *HPX* and *POTEE* genes by omic analysis in breast cancer

NACI CINE[1], AHMET TARIK BAYKAL[2], DENIZ SUNNETCI[1], ZAFER CANTURK[4], MUGE SERHATLI[3] and HAKAN SAVLI[1]

[1]Department of Medical Genetics, Faculty of Medicine, Kocaeli University, Kocaeli; [2]Department of Medical Biochemistry, School of Medicine, Istanbul Medipol University, Istanbul; [3]Genetic Engineering and Biotechnology Institute, Marmara Research Center, TUBITAK, Kocaeli; [4]Department of General Surgery, Faculty of Medicine, Kocaeli University, Kocaeli, Turkey

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Abstract. Breast cancer is the most common cancer among women and accounts for 23% of all female types of cancers. It is well recognized that breast cancer represents a heterogeneous group of tumors, and the molecular events involved in the progression to cancer remain undetermined. Moreover, available prognostic and predictive markers are not sufficient for the accurate determination of the risk for many breast cancer patients. Thus, it is necessary to discover new molecular markers for accurate prediction of clinical outcome and individualized therapy. In the present study, we performed omics-based whole-genome trancriptomic and whole proteomic profiling with network and pathway analyses of breast tumors to identify gene expression patterns related to clinical outcome. A total of 20 samples from tumors and 14 normal appearing breast tissues were analyzed using both gene expression microarrays and LC-MS/MS. We identified 585 downregulated and 413 upregulated genes by gene expression microarrays. Among these genes, *HPX*, *POTEE* and *ApoA1* were the most significant genes correlated with the proteomic profile. Our data revealed that these identified genes are closely related to breast cancer and may be involved in robust detection of disease progression.

Introduction

Breast cancer is the most common type of cancer among women, accounting for ~10% of all types of cancer diagnosed annually and 23% of all new cancer cases worldwide. It was estimated that 1.4 million new cases were diagnosed in 2008. More than a 13-fold increase was globally estimated in the incidence of female breast cancer in 2008 (1,2). In addition, breast cancer is an extremely common type of cancer in Turkey, which is consistent with worldwide statistics. The estimated number of breast cancer cases was 44,253 in 2007 in Turkey (3,4). The incidence of the disease is 20% in women younger than 40 years, while it has been estimated to be approximately 5% in Western Europe and the US (5). The pathophysiology of breast cancer is highly complex, and several factors have been reported to be associated with the development and prognosis of the disease, including genetic, hormonal, environmental, sociobiological and physiological factors (6,7). Targeting the underlying causes of breast cancer will offer a better understanding of the etiology of the disease. The introduction of novel management approaches based on recent technological developments is also highly useful to investigate the underlying causes of such diseases with a heterogeneous nature. Omics-based recent approaches, in particular, have allowed an integrated evaluation of the system and have elucidated the underlying mechanisms behind the disease. Omics technology encompasses high-throughput assays of major systems such as genomics, proteomics and metabolomics. Whole genomic and proteomic studies have demonstrated that the major and minor factors which play a role in the development of complex diseases are likely to be assessed concomitantly (8,9). In addition, studies integrating genomic, proteomic and metabolomic datasets have provided further information concerning systems biology, allowing us to accurately identify the underlying factors and evaluate the functions within a conceptual framework.

In the present study, we aimed to investigate biomarkers which play a role in the development of breast cancer using tissue samples through omics-based whole-genome trancriptomic and whole proteomic profiling. The present study results demonstrated that hemopexin (*HPX*), prostate, ovary, testis-expressed protein E (*POTEE*), apolipoprotein A1 (*Apo-A1*), matrix metalloproteinase-9 (MMP-9) molecule and nuclear factor-κB (NF-κB) gene networks may play a role in the etiology of this disease. We believe that significant differences in *HPX*, *POTEE* and *Apo-A1* molecules, particularly in whole-genome trancriptomic and whole proteomic studies advocate further research on these molecules.

Correspondence to: Dr Naci Cine, Department of Medical Genetics, Faculty of Medicine, Kocaeli University, Kat-1 Umuttepe, 41380 Kocaeli, Turkey
E-mail: naci.cine@kou.edu.tr

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Materials and methods

Tissue collection. Tissue samples from malignant tumors and their normal counterparts were obtained from patients who underwent surgery for breast cancer at the Department of General Surgery of Kocaeli University in the period 2009-2010. Tissue samples were snap-frozen immediately and then stored at -80˚C. A total of 20 samples from tumors were analyzed. Most of the samples were invasive ductal carcinomas (12 samples) and others were invasive lobular (1 sample), ductal carcinoma *in situ* (1 sample) and invasive micropapillary carcinoma (1 sample). The mean patient age at surgery was 52.8 years with a median age of 52 years (range 38-73). Tumors were evaluated by a breast pathologist to confirm the diagnosis. ER, PR and c-erbB2 status were determined by immunohistochemistry (IHC) at the Department of Pathology, Kocaeli University. Nine samples were estrogen-positive, 5 samples were progesterone-positive and 8 samples were positive for c-erbB2. Fourteen normal appearing breast tissues were obtained from women undergoing surgery. The mean age of these women was 54.2 years, with a median age of 56 years (range 38-73). The study was approved by the Kocaeli University Ethics Committee.

Total RNA isolation. Frozen breast tissues were sectioned for RNA extraction. Total RNA was isolated from cells of each patient using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) and treated with DNase I according to the manufacturer's instructions. Sample purity was confirmed by measuring A260/A280 ratios. The quality of the RNA was assessed by loading 300 ng of total RNA onto an RNA LabChip, followed by analysis (Agilent 2100 Bioanalyzer) (both from Agilent Technologies, Waldbronn, Germany). An RNA integrity value (RIN) of ≥6.10 was considered as acceptable.

Microarray and statistical analysis. Microarray analysis was performed using the Whole Human Genome Oligo Microarray (Agilent Technologies), encompassing >44,000 human DNA probes. The full list of cDNAs is available online (http://www.agilent.com). Protocols for sample preparation and hybridization of the cells were adaptations of those in the Agilent technical manual. Briefly, first strand cDNA was transcribed from 200 ng of total RNA using T7-Oligo(dT) promoter primer. Samples were transcribed *in vitro* and Cy-3-labeled using a Quick Amp labeling kit (Agilent Technologies). Following a further clean-up round (Qiagen), cRNA was fragmented into pieces ranging from 35 to 200 bases in size. Fragmented cRNA samples (1.65 mg) were hybridized onto chips by means of 17 h of incubation at 65˚C with constant rotation, followed by a two-step microarray wash of 1 min in two washing buffers (Agilent Technologies). Hybridized microarrays were scanned in an Agilent Technologies scanner (model G2505B), and numerical results were extracted with Feature Extraction version 9.5.1.1 using 014850_D_F_20060807 grid, GE1-v5_95_Feb07 protocol and GE1_QCM_Feb07 QC metric set. The microarray data were analyzed using GeneSpring software version 11.0 (Agilent Technologies, Santa Clara, CA, USA). The fold-changes were analyzed by filtering the dataset using P-value <0.05 and a signal-to-noise ratio ≥2 for use in t-test statistical analysis. Additional filtering (minimum 2.00-fold-change) was applied to extract most genes, which were analyzed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). Those genes with known gene symbols (HUGO) and their corresponding expression values were uploaded into the software. Each gene symbol was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Networks of these genes were algorithmically generated based on their connectivity and assigned a score. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset but may not be an indication of the quality or significance of the network. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes. The network identified was then presented as a graph indicating the molecular relationships between genes/gene products. Genes were represented as nodes, and the biological relationship between two nodes was represented as an edge (line). The intensity of the node color indicates the degree of upregulation or downregulation. The node shapes are provided in corresponding legends. Canonical pathway analysis identified the pathways from the IPA library of canonical pathways, which were most significant to the input dataset. The significance of the association between the dataset and the canonical pathway was determined based on two parameters: i) a ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the canonical pathway; and ii) a P-value calculated using Fischer's exact test determining the probability that the association between the genes in the dataset and the canonical pathway is due to chance alone.

Quantitative real-time PCR (qRT-PCR). cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Fermentas Inc., Glen Burnie, MD, USA). qRT-PCR was performed as described previously for determination of *Apo-A1* and *HPX* gene expression. Standard curves were obtained using serial dilutions of the β-globulin gene (DNA Control kit; Roche). Gene-specific primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Obtained gene expression values were normalized using a housekeeping gene of β2 microglobulin. Gene expression ratios were compared in patient and control groups using relative expression software tool (REST).

Proteomic analysis

Extraction and trypsin digestion of proteins. Tissue samples were frozen with liquid nitrogen and pulverized with a bead beater (Retsch MM301). UPX extraction buffer (500 μl) (Expedeon) spiked with 5 μl protease inhibitor cocktail (Sigma-Aldrich) was added to the samples and boiled at 100˚C for 5 min and subsequently centrifuged at 14,000 rpm for 15 min to remove debris. The supernatant was transferred to a clean Eppendorf tube, and protein concentration measurement was carried out with a Nanodrop spectrophotometer at a wavelength of 280 nm (Thermo ND-1000). Tryptic peptides were generated according to the filter-aided sample preparation protocol (FASP) (1). Briefly, 50 mg protein was washed with 6 M urea in a 30-kDa cut-off spin column and then alkylated with 10 mM iodoactamide (IAA) in the dark at

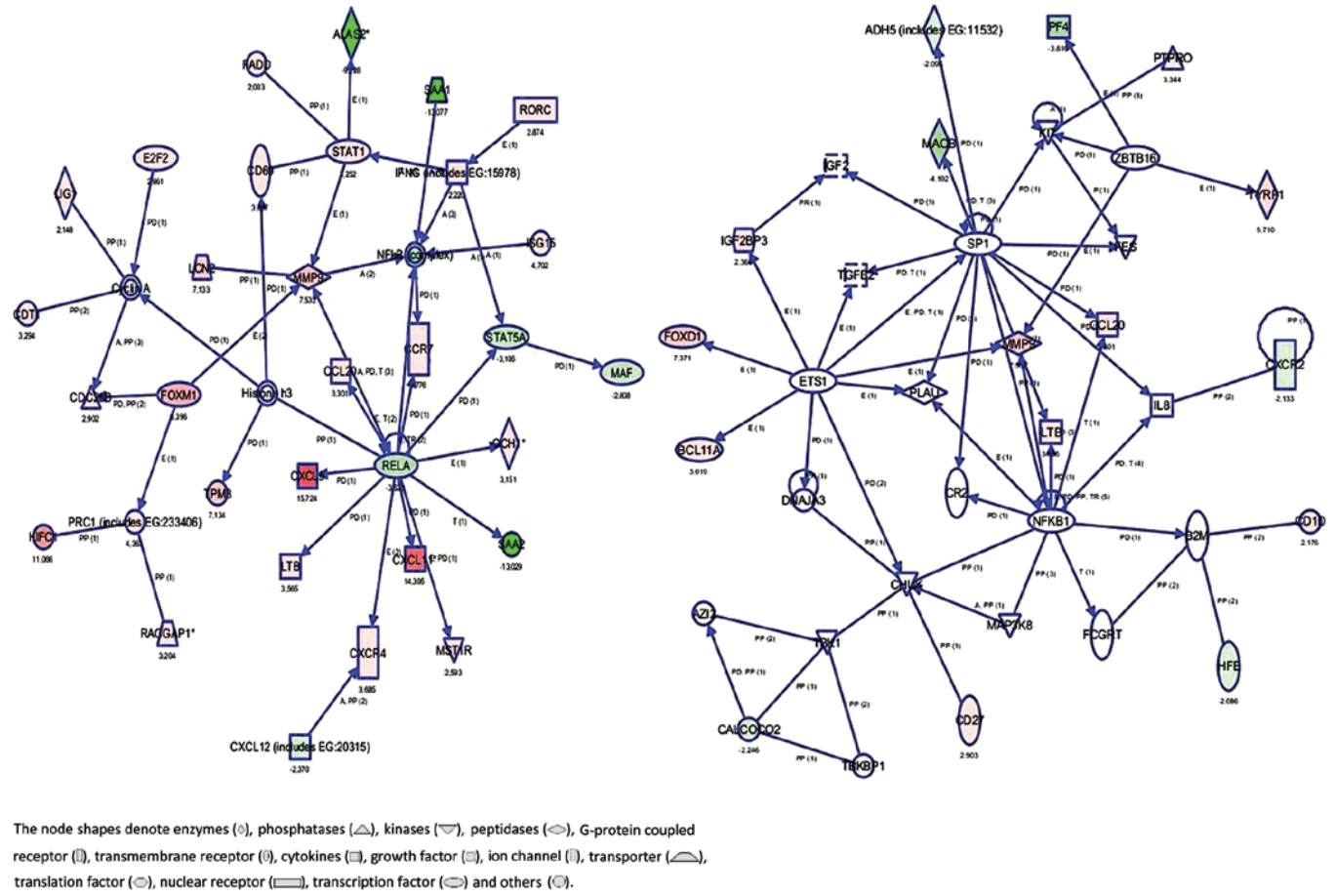

Figure 1. Significantly upregulated and downregulated genes were identified around the *NFκB* and *MMP-9* gene networks. Genes are represented as nodes, and the biological relationship between two nodes are represented as an edge (line). The intensity of the node color indicates the degree of upregulation or downregulation. Genes in uncolored nodes were not identified as differentially expressed in our experiment and were integrated into the computationally generated networks based on the evidence stored in the IPA knowledge memory indicating relevance to this network.

room temperature for 20 min. Subsequently, the samples were first washed with 6 M urea to remove IAA and later with ammonium bicarbonate solution to remove the urea from the samples and finally tripsinized overnight (1:100, trypsin to protein ratio). Peptides were eluted from the spin column, and the concentration was measured with a Nanodrop spectrometer and adjusted to a concentration of 100 ng/μl and spiked with 50 fmol internal standard (MassPREP Enolase Digestion Standard; Waters, Milford, MA, USA).

LC-MS/MS analysis and database search. The LC-MS/MS analysis and protein identifications were carried out according to a previously published protocol (2). Briefly, 500 ng tryptic peptides in 5 μl for each experimental condition was analyzed by the nLC-MS/MS system [(nanoAcquity ultra pressure liquid chromatography (UPLC) and Synapt high definition mass spectrometer with NanoLockSpray ion source (Waters)]. Columns were equilibrated with 97% mobile phase A [0.1% formic acid in LC-MS grade water (Merck)] and the column temperature was set to 45˚C. Peptides were separated from the trap column (Symmetry C18, 5 μm, 180 μm i.d. x 20 mm) by gradient elution onto an analytical column (BEH C18, 1.7 μm, 75 μm i.d. x 250 mm) (both from Waters) at 300 nl/min flow rate with a linear gradient from 5 to 40% mobile phase B [0.1 formic acid in hypergrade acetonitrile (Merck)] over 90 min.

Data independent acquisition mode (MS^E) was carried out by operating the instrument at positive ion V mode, applying the MS and MS/MS functions over 1.5 sec intervals with 6 V low energy and 15-40 V high energy collusion. Glu-fibrinopeptide (internal mass calibrant) was infused every 45 sec at 300 nl/min flow rate. m/z values over 50-1,600 were recorded. Tandem mass data extraction, charge state deconvolution and deisotoping were carried out with ProteinLynx Global Server v2.5 (Waters) and searched with the $IDENTITY^E$ algorithm with a fragment ion mass tolerance of 0.025 Da and a parent ion tolerance of 0.0100 Da against the reviewed *Homo sapiens* protein database from Uniprot (June 1, 2012, 25,899 entries). The amino acid sequence of the internal standard (yeast enolase, Uniprot accession no: P00924) was included in the FASTA file of the database. The Apex3D data preparation parameters were set to 0.2 min chromatographic peak width, 10.000 MS TOF resolution, 150 counts for low energy threshold, 50 counts for elevated energy threshold, and 1,200 counts for the intensity threshold. Databank search query was set to minimum 3 fragment ion matches/peptide, minimum 7 fragment ion matches/protein, minimum 1 peptide matches/protein and 1 missed cleavage. Carbamidomethyl-cysteine fixed modification and N-terminal acetyl, deamidation of asparagine and glutamine, and oxidation of methionine variable modifications

Table I. Selected genes were detected as upregulated in the whole genome expression array dataset.

Gene symbol	Fold-change (Tumor vs. normal)	Log fold-change (Tumor vs. normal)	Absolute fold-change (Tumor vs. normal)	Regulation (Tumor vs. normal)	Description
CXCL9	15.723808	3.9748788	15.723808	Up	Chemokine (C-X-C motif) ligand 9, [mRNA NM_002416]
KIFC1	11.06615	3.4680815	11.06615	Up	Kinesin family member C1, mRNA [NM_002263]
FOXM1	9.396099	3.2320619	9.396099	Up	Forkhead box M1, mRNA [NM_202002]
MMP-9	7.5315833	2.9129531	7.5315833	Up	Matrix metallopeptidase-9, mRNA [NM_004994]
TPM3	7.133844	2.8346796	7.133844	Up	Tropomyosin 3, mRNA [NM_001043352]
HPX	5.06874	2.3416271	5.06874	Up	Hemopexin, mRNA [NM_000613]
RRM2	3.7532458	1.9081388	3.7532458	Up	Ribonucleotide reductase M2, mRNA [NM_001034]
E2F2	2.6612885	1.4121249	2.6612885	Up	E2F transcription factor 2, mRNA [NM_004091]
SREBF1	2.3270373	1.2184944	2.3270373	Up	Sterol regulatory element binding transcription factor 1, mRNA [NM_001005291]
POTEE	2.3189814	1.2134912	2.3189814	Up	POTE ankyrin domain family member E, mRNA [NM_001083538]

Up, upregulated.

were set. Quantification of the protein expression changes was carried out with Progenesis LC-MS software v4.0 (Nonlinear Dynamics). Normalization across the sample set was based on total ion signal. Protein quantification was carried out with only the non-conflicting peptide features.

Results

Microarray analysis. Differentially expressed genes were determined as upregulated or downregulated. After data analysis, we identified 585 downregulated and 413 upregulated genes. A selected 10 upregulated and downregulated genes are shown in Tables I and II. Both sets of results were obtained based on a minimum 2.00-fold-change using GeneSpring software version 11.0 (Agilent Technologies). The gene expression results of 3 genes; *HPX*, *POTEE* and *Apo-A1*, performed by real-time PCR and microarray methods were verified. We investigated interactions using IPA software and found 4 gene networks including both downregulated and upregulated genes. Fig. 1 shows the most significant 4 gene networks in the breast cancer samples. Top functions of these genes were found to be related to inflammatory response, hematological system development and function, hematopoiesis, genetic disorder, hematological disease, cancer, embryonic development, lymphoid tissue structure and development, organ development, tissue morphology, cellular growth and proliferation. These gene networks are identified around *NF-κB1* and *MMP-9*. Table III lists the top 5 canonical pathways including downregulated and upregulated genes.

Proteomic analysis by label-free LC-MS/MS. Label-free shotgun proteomic analysis was applied to compare the differential proteome expression of the cancer and control samples. Typical coefficient of variations observed for the nanoLC-MS/MS analysis with the nanoAcquity UPLC system coupled to a Synapt high definition mass spectrometer was ~10-14%. Only protein expression changes above 40% were considered to be statistically significant. Thirty-four tissue samples were homogenized, proteins extracted and analyzed by nanoLC/MS/MS and 683 proteins in 357 protein groups were identified and 291 proteins out of 357 were quantified. Power analysis was also performed for the analysis that indicated whether the sample set had enough replicates to determine real differences among the sample groups. The data (89.5%) had a power value of >0.8, indicating that the number of replicates in each group was satisfactory to represent the group. One hundred and forteen proteins showed >40% expression change and were statistically significant. We were most interested in 4 of the statistically significantly altered proteins. Apo-A1, POTEE and HPX were identified with 28, 36 and 8 unique peptide sequences, respectively. Protein quantification was based on non-conflicting features and Apo-A1, POTEE and HPX were quantified based on 14.2 and 5 peptide sequences, respectively. Given the high number of unique peptide sequences identified

Table II. Selected genes were detected as downregulated in the whole genome expression array dataset.

Gene symbol	Fold-change (Tumor vs. normal)	Log fold-change (Tumor vs. normal)	Absolute fold-change (Tumor vs. normal)	Regulation (Tumor vs. normal)	Description
APOA1	-2.0003383	-1.000244	2.0003383	Down	Apolipoprotein A-I, mRNA [NM_000039]
CSDA	-2.0459063	-1.03274	2.0459063	Down	Cold shock domain protein A, transcript variant 1, mRNA [NM_003651]
NFAT5	-2.128091	-1.0895599	2.128091	Down	Nuclear factor of activated T-cells 5, tonicity-responsive, transcript variant 1, mRNA [NM_138714]
MSC	-2.1575832	-1.1094162	2.1575832	Down	Musculin (activated B-cell factor-1), mRNA [NM_005098]
NRP2	-2.1909428	-1.1315519	2.1909428	Down	Neuropilin 2, transcript variant 6, mRNA [NM_201264]
LMO2	-2.2316236	-1.1580938	2.2316236	Down	LIM domain only 2 (rhombotin-like 1), transcript variant 1, mRNA [NM_005574]
CXCL12	-2.3697665	-1.2447449	2.3697665	Down	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1), transcript variant 2, mRNA [NM_000609]
MAF	-2.808239	-1.4896657	2.808239	Down	V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian), transcript variant 2, mRNA [NM_001031804]
STAT5A	-3.106449	-1.6352663	3.106449	Down	Signal transducer and activator of transcription 5A, mRNA [NM_003152]
RELA	-3.5231855	-1.8168805	3.5231855	Down	V-rel reticuloendotheliosis viral oncogene homolog A (avian), transcript variant 2, mRNA [NM_001145138]

Down, downregulated.

for each protein, it was confirmed that the protein identification was stringent and accurate (Table IV).

Discussion

In parallel with various types of studies, the primary goal of cancer research is to understand the underlying mechanisms of the disease. It has been well-established that cancer involves several mechanisms including cell proliferation, differentiation, apoptosis and inflammation (10-12). Each of these mechanisms has a highly complex gene-protein relationship. Major mutations may induce the development of cancer, while minor factors with sequential networks and pathways may contribute to the development of the disease (13-16). High-throughput assays offer several advantages in such diseases with a heterogeneous nature. Recent omics-based approaches, in particular, integrate expression and proteomic and metabolomics data, allowing us to assess major and minor diseases concomitantly (9,17).

In the present study, we evaluated breast cancer within the framework of omics science. We performed whole-genome expression array analysis and proteomic studies using the same tissue samples obtained from patients with breast cancer. In the present study, we observed significant networks

Table III. Gene set enrichment analysis.

Pathway	-Log (P-value)	Ratio	Molecules
LXR/RXR activation	1.00 E-03	9.50 E-02	AMBP, APOA1, APOA4, APOC1, HPX, MMP-9, RELA, SAA1, SAA2, SAA4, SCD, SREBF1
PKCθ signaling in T lymphocytes	5.43 E-04	9.40 E-02	CARD11, CD3D, HLA-DOA, HLA-DQA1, RAC2, RELA, SOS1, ZAP70
T cell receptor signaling	4.79 E-04	1.08 E-01	BMX, CARD11, CD3D, LCK, NFAT5, PIK3CD, PTPRH, RASGRP1, RELA, SOS1, ZAP70
Atherosclerosis signaling	1.89 E-04	1.04 E-01	APOA4, APOC1, COL10A1, CXCL12, CXCR4, IFNG, MMP-9, PLA2G10, PLA2R1, RELA, SAA4
CTLA4 signaling in cytotoxic T lymphocytes	6.17 E-05	1.26 E-01	AP1G2, AP1M2, CD3D, HLA-DOA, HLA-DQA1, LCK, PIK3CD, PPP2CB, PPP2R2C, PTPN22

The nominal P-value estimates the statistical significance of the enrichment score for a single gene set. Significant false discovery rate (FDR) and nominal P-values were <5% and 0.05, respectively.

Table IV. Protein profiling studies performed in control and breast cancer tissues by label-free shotgun proteomic analysis.

Protein	Peptides	Score	ANOVA (P-value)	Fold	Description	Average normalized abundance	
						Cancer	Control
EF1A3	27 (1)	160.51	1.13E-05	3.52	EF1A3 HUMAN putative elongation factor 1α like 3 OS *Homo sapiens* GN EEF1A1P5 PE 5 SV 1	1571.57	446.63
CAH1	7 (4)	72.74	5.35E-06	2.69	CAH1 HUMAN carbonic anhydrase 1 OS *Homo sapiens* GN CA1 PE 1 SV 2	2240.24 515.9	6018.94 201.04
POTEE	36 (2)	246.87	6.94E-08	2.57	POTEE HUMAN POTE ankyrin domain family member E OS *Homo sapiens* GN *POTEE* PE 1 SV 3		
ACTG	44 (2)	322.46	9.20E-09	2.51	ACTG HUMAN actin cytoplasmic 2 OS *Homo sapiens* GN ACTG1 PE 1 SV 1	1952.78	778.64
Hemopexin	8 (5)	68.2	4.88E-08	2.11	HEMO HUMAN hemopexin OS *Homo sapiens* GN HPX PE 1 SV 2	3280.94	1552.96
IGHA2	10 (1)	70.72	4.87E-06	1.88	IGHA2 HUMAN Ig α 2 chain C region OS *Homo sapiens* GN IGHA2 PE 1 SV 3	1076.4	2020.74
IGHG2	22 (4)	135.6	1.59E-05	1.83	IGHG2 HUMAN Ig γ 2 chain C region OS *Homo sapiens* GN IGHG2 PE 1 SV 2	4238.46	7765.97
APOA1	28 (14)	209.27	1.14E-06	1.77	APOA1 HUMAN apolipoprotein AI OS *Homo sapiens* GN APOA1 PE 1 SV 1	1.62E+04	2.87E+04
IGHA1	13 (4)	90.63	2.80E-06	1.69	IGHA1 HUMAN Ig α1 chain C region OS *Homo sapiens* GN IGHA1 PE 1 SV 2	3917.9 38.28	6617.12 24.95
POTEF	32 (1)	215.04	0.01	1.53	POTEF HUMAN POTE ankyrin domain member F OS *Homo sapiens* family GN POTEF PE 1 SV 2		

and demonstrated a significant correlation among, *HPX* and *POTEE* molecules using both array and proteomic data.

Apolipoprotein A-1 (*Apo-A1*) is the major protein component of high density lipoprotein (HDL) (18-20). It is synthesized mainly in the liver and small intestine (21). As confirmed by several studies, *Apo-A1* suppresses inflammation, tumor growth, angiogenesis, invasion and metastasis (22). Studies have found that *Apo-A1* is a potential biomarker for

many types of cancer including breast and pancreatic carcinomas (23-27). It is usually thought that the role of the *Apo-A1* molecule in cancer pathophysiology is associated with the phospholipid binding ability. Lysophospholipids, in particular, have been shown to play a critical role in the development of cancer and have been reported to be major biomarkers for cancerous diseases (28,29). Animal studies have also shown that overexpression of the *Apo-A1* gene led to the inhibition of tumor growth in mice and prolonged survival (30). In the present study, we found reduced *Apo-A1* gene expression in the patients with breast cancer as determined by microarray analyses. We also found reduced *Apo-A1* in tumor tissues based on proteomic studies. Our study results which were consistent with the literature data indicate that reduced *Apo-A1* may play a role in the development of tumors. The examination of tissue samples obtained from the patients with breast cancer also suggested that *Apo-A1* may play an active role in the development of breast cancer. In addition, it is well-known that *Apo-A1*, an anti-apoptotic and antioxidant molecule, functions in transporting other antioxidant molecules and may directly affect the intracellular signaling pathways (27,31). Similarly, we confirmed that *Apo-A1* is a critical candidate biomarker for the development of cancer. We also observed a significant increase in *MMP-9* gene expression according to the microarray analyses. This finding, which indicates the role of *MMP-9* alone and in combination with *Apo-A1* in the development of cancer, is important. *Apo-A1* was found to inhibit *MMP-9* (30). The *MMP-9* is a zinc-dependent endopeptidase which is responsible for the degradation of the extracellular matrix (32). It plays a major role in cellular behaviors such as tumor angiogenesis, invasion and metastasis. Higher levels of active *MMP-9* were observed in highly invasive and metastatic melanoma tumors (33-35). Degradation of the extracellular matrix results in invasion and metastasis of the disease. Matrix metalloproteinases are synthesized by epithelial and mesenchymal cells including leukocytes, keratinocytes, fibroblasts, macrophages, chondrocytes and smooth muscle cells (36-38). MMP expression was found in endometrial vessels during the involution of mammary glands and inflammation in adult women. MMPs play an important role in the pathology of many diseases, although their roles in the underlying mechanisms of cell proliferation and differentiation, remodeling, ovulation, cellular migration and angiogenesis remain to be elucidated (39). MMPs, which are initially secreted in the form of inactive zymogens, are inhibited by specific tissue inhibitors (TIMPs) and *Apo-A1* (35). *MMP-9* is one of the matrix metalloproteinases which is mostly found in malignant tissues with high tumor aggressiveness and metastatic potential (32,33). In the present study, we obtained similar results with reduced *Apo-A1* expression and increased *MMP-9* expression. *MMP-9* was previously shown to be increased in plasma and urinary samples, as well as cancer tissues in patients with breast cancer (33,38,39). In the present study, we also observed overexpression of *MMP-9*, which is consistent with the literature data. It is known that *MMP-9* is inhibited by *Apo-A1* in healthy tissues. However, we found an inverse relationship between *MMP-9* inhibition and *Apo-A1* with a significant difference between the breast cancer and healthy tissues. These findings were inconsistent with the literature data, suggesting the critical role of the relationship between *MMP-9* and *Apo-A1* in the development of breast cancer. According to the literature data, it can be concluded that the ability of *Apo-A1* to inhibit *MMP-9* is deactivated in the development of breast cancer without negative control for *MMP-9* through *Apo-A1*.

In the present study, we assessed all data at the network level. We aimed to investigate upregulated and downregulated networks and identify major genes. The presence of the *NF-κB* gene family and *MMP-9* gene in both upregulated and downregulated analyses is important. Based on the upregulated analysis data, we found a large-sized and integrated gene network in which *REL-A* was in the center. Overexpressed *MMP-9* was associated with this gene network. Based on the downregulated analysis data, we found a gene network in which the NF-κB1 gene was relatively in the center with overexpressed *MMP-9*. *NF-κB* is a transcription factor which plays a major role in immunity, cell proliferation, cell survival and cancer (40). Activation of *NF-κB* is increased in many types of cancers, and is associated with various steps in the development of malignancy, such as expression of anti-apoptotic genes, angiogenesis, tumor growth and metastasis (41,42). *NF-κB* regulates several genes such as cyclooxygenase-2 (*COX-2*), nuclear factor-κB inhibitor α (*IκBα*), tumor necrosis factor α (*TNF-α*), cyclin D1, intercellular adhesion molecule 1 (*ICAM-1*), *c-myc*, *Bcl-2*, inducible nitric oxide synthase (*iNOS*), and interleukins including *IL-6* and *IL-8* and also *MMP-9* (43-45). The present study results were consistent with previous study findings which indicate the role of *NF-κB* in the development of cancer. All data obtained from the array and proteomic data along with the network analysis suggest that *MMP-9,* in particular, is a critical player in the development of breast cancer. We also found a significant relationship between *MMP-9,* and gene expression of other genes in the network. Our findings highlighted the crucial role of *NF-κB* in the gene network analyses in the development of breast cancer.

In the present study, *HPX* was another molecule which was consistent with the proteomic and transcriptomic data. We observed that *HPX* was upregulated based on the whole-genome cDNA array and proteomic analyses. The human *HPX* protein is a 60-kDa plasma glycoprotein, which consists of two domains joined by a linker sequence (46). *HPX* represents the primary line of defense against heme-related oxidative stress and toxicity (47). The *HPX* molecule is capable of binding heme with high affinity and acting as a heme-specific carrier from the bloodstream to the liver (48). Heme, which is the functional group of diverse hemoproteins, is critical for many cellular processes. Excessive free heme may be detrimental to tissues by mediating oxidative and inflammatory injury (46,49). Murrell demonstrated that, similar to free oxygen radicals, chemical carcinogens may also result in damage to mammary epithelial cells, fibroblastic proliferation, epithelial hyperplasia, cellular atypia and, thereby, breast cancer (50). Similarly, an increased *HPX* rate may be an indicator of actual oxidative stress in the breast tumors in this study. Experimental and epidemiological studies have shown that free oxygen radicals play a role in the pathogenesis of cancer (50). Free oxygen radicals, in the presence of metal ions particularly, may lead to oxidative damage, interacting with cellular proteins, lipids and macromolecules with DNA. Improved defense mechanisms with antioxidants may reduce oxidative damage. It is well-established that molecules

such as heme oxygenase-1 (HO-1) and glucose-6-phosphate dehydrogenase (G6PD) act as antioxidants against free oxygen radicals and offer a cellular defense against oxidative stress (51,52). In the present study, overexpressed *HPX* may be rationalized by the cellular defense of this molecule, similar to HO-1 and G6PD. We believe that *HPX* was overexpressed due to oxidative stress during the development of breast cancer, exhibiting a preventive role against oxidative damage with its cellular defense mechanism. Overexpression of *HPX* may be an indicator of actual oxidative stress in breast cancer. As a result, a novel diagnostic approach based on the prediction of oxidative damage using several molecules including *HPX* may be developed for patients with breast cancer.

In the present study, *POTEE* was another molecule which was significantly increased based on both array and proteomic data. Recent studies suggest that *POTEE* family proteins may be involved in signal transmission across the plasma membrane. *POTEE* was initially found to be expressed in normal prostate, ovary, testis and placenta tissues, as well as in prostate cancer (53). Recently, *POTEE* was identified in a variety of human cancers as a novel tumor-associated antigen (53-55). POTE was shown to be expressed in a wide variety of human cancers (53). Published studies revealed that *POTEE* genes were expressed not only in prostate cancer, but also in a wide variety of human malignancies, including breast, colon, lung, ovaran and pancreatic cancer (54). The amount of *POTEE* molecules which are produced is very low in normal tissues. Increased expression of these molecules has been shown in prostate and breast cancer (54,55). Low levels of these molecules in normal tissues and higher levels in tumor cells alone make the *POTEE* molecule a critical immunotherapeutic biomarker (56). It is thought that *POTEE* can be a suitable antibody for radiotherapy and immunotherapy in patients with cancer, as it is only increased in cancer tissues. In the present study, we observed *POTEE* overexpression based on the whole-genome expression array analysis and proteomic studies using breast cancer tissue samples. This finding, consistent with previous study findings, supports the opinion that *POTEE* protein is a critical player in breast cancer. On the other hand, there is a limited number of studies indicating increased expression of *POTEE*. We believe that our study contributes to the literature. In addition, we demonstrated that increased expression of *POTEE* was accompanied by increased gene expression. Therefore, novel diagnostic and monitoring technologies should be developed within the framework of gene expression, as well as antibody therapy.

In conclusion, in the present study, which was conducted in the light of the literature data, we found that *MMP-9*, in particular, and its biological mechanisms are of utmost importance for breast cancer. Identification of *Apo-A1* and overexpression of *MMP-9* in both the array and proteomic studies underline the role of *Apo-A1* and *MMP-9* in the pathophysiology of the disease. These findings also highlight the importance of omics-based approaches in elucidating the underlying mechanisms behind complex diseases such as cancer. Previous studies implicate these three molecules, *Apo-A1*, *HPX* and *POTEE*, in the development of breast cancer. The present study results were also consistent with previous findings, highlighting their major role in the underlying pathology. *Apo-A1*, in particular, was associated with breast cancer and is considered to be a critical player in the development of the disease due to its molecular characteristics. *POTEE* and HPX, in contrast, are indicators of existing pathology and may be considered for further diagnostic and therapeutic applications. We conclude that molecular targeting studies may produce significant improvements in the diagnosis and treatment of breast cancer.

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