Expression of Matrix Metalloproteinases (MMPs) in Primary Human Breast Cancer: MMP-9 as a Potential Biomarker for Cancer Invasion and Metastasis

ADNAN MERDAD[1*], SAJJAD KARIM[2*], HANS-JUERGEN SCHULTEN[2], ASHRAF DALLOL[2], ABDELBASET BUHMEIDA[2], FATIMA AL-THUBAITY[1], MAMDOOH A. GARI[2], ADEEL GA CHAUDHARY[2], ADEL M ABUZENADAH[2] and MOHAMMED H. AL-QAHTANI[2]

[1]*Department of Surgery, King Abdulaziz University Hospital, King Abdulaziz University, Jeddah, Saudi Arabia;*
[2]*Center of Excellence in Genomic Medicine Research, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia*

Abstract. *Background/Aim: Breast cancer (BC) is the most common type of cancer in Saudi women. Matrix metalloproteinases (MMPs) are endopeptidases with the ability to degrade extracellular matrix proteins. In healthy individual tissue disruption is prevented by precised regulation of MMPs; however, in cancer a number of MMPs are overexpressed causing tissue disruption and making tumor cells capable of invasion and metastasis. Invasive ductal carcinoma (IDC) of BCs are classified into grade 1 (G1), grade 2 (G2) and grade 3 (G3) tumors. Materials and Methods: We performed a transcriptomic profiling of 38 surgically-resected breast tumors (4 G1, 17 G2 and 17 G3) using Affymetrix Gene 1.0 ST microarrays. Differentially expressed genes for each grade were identified by the Partek Genomic Suite 6.4 and expression analysis results were validated by immunohistochemistry at the protein level. Pathway analyses and establishment of clinical significance of findings were performed using the appropriate software. Results: We identified 1,593 differentially expressed genes in BC grades in comparison to normal samples using a cut-off of $p<0.05$ and fold change >2. Out of these genes 429 were expressed throughout in all grades along with tumor progression while many others associated with specific grades (440 genes in G1, 203 in G2 and 394 in G3 only) were exclusively. Microarray results indicate that mRNA expression of MMP-1, -9,-11,-12, and -13 were up-regulated in higher BC grades when compared to normal breast tissues. MMP-9 was expressed in most IDC (97.5%) samples and was highly expressed in 55% of the tumors. Differential expression of MMP-9 significantly correlated with histological BC grades of ($p=0.03$) and strongly correlated with overall survival ($p=0.08$). Conclusion: Gene expression signatures are unique for specific grades. Overexpression of MMPs in higher grades might be associated with BC tumor invasion and metastasis. Therefore, MMPs, and MMP-9 in particular, are reliable candidates for diagnostic biomarker and drug target and further functional analyses have to be performed in order to confirm their role in BC. Our results also suggest the incidence of MMP-9 expression is high in IDC, but it is of limited prognostic value.*

Breast cancer (BC) is the most common type of cancer in women around the world, including Saudi Arabia and is among the leading causes of cancer-related mortality (1-3). It is estimated that more than one million new cases of BC are diagnosed annually worldwide (4). According to the Saudi cancer registry, incidence of BC in women is high (25.1%), affecting mainly women under the age of 50, while in the U.S.A., women older than 50 years of age are most frequently affected (4, 5).

Based on origin, BC is classified into two main types, namely ductal carcinoma (that starts in the cells of milk ducts, the tubes that transport milk to the nipple) and lobular carcinoma (that starts in the breast lobules, the milk producing glands). BC can further be divided into invasive (spread) or non-invasive (*in situ*) types (6). Invasive ductal carcinoma (IDC) or infiltrating ductal carcinoma is the most

*These Authors contributed equally to this work.

Correspondence to: Dr. Sajjad Karim, Center of Excellence in Genomic Medicine Research, King Abdulaziz University, PO BOX 80216, Jeddah 21589, Kingdom of Saudi Arabia. Tel: +966 126401000 ext 25123, Fax: +966 126952521, e-mail: skarim1@kau.edu.sa; sajjad_k_2000@yahoo.com and Dr. Mohammed H. Al-Qahtani, Director, Center of Excellence in Genomic Medicine Research, King Abdulaziz University, PO BOX 80216, Jeddah 21589, Kingdom of Saudi Arabia. Tel: +966 126401000 ext 25962, Fax: +966 126952521, e-mail: mhalqahtani@kau.edu.sa

Key Words: Breast cancer, invasive ductal carcinoma, histological grade, gene expression, MMPs, MMP-9, Saudi Arabia.

common type of BC, and accounts for 70-80% of breast cases (7). Additionally, according to histological features, invasive BCs can be classified into three grades: grade 1 (well-differentiated), grade 2 (moderately-differentiated) and grade 3 (poorly-differentiated) tumors. IDC begins in a breast milk duct, infiltrates the wall of the duct and invades nearby tissues. After the invasion, IDC has the potential to metastasize to other parts or organs in the body through the lymphatic system or bloodstream and distant metastases are the principal cause of death. Degradation of the extracellular matrix is an essential process allowing tumor cells to invade local tissue and blood vessels and move to new metastatic location. Tumor cells secrete specific proteinases; known as serine proteinases, aspartatic proteinases, cysteine proteinases and matrix metalloproteinases that primarily influence tumor invasion and metastasis (8-10). In 2010, the estimated number of invasive breast cancer cases in the USA was 207,090 and out of this number 40,000 women were expected to die of cancer (11). Thus exigent approaches are requisited to discover biomarkers for better screening, diagnosis, prognosis and treatment of BC.

Transcriptomics approaches that allow for screening of thousands of genes in a single experiment, have had a major impact on BC research over the past 10 years (12). Several groups have carried-out gene expression profiling of BC to classify clinically-distinct sub-classes of sporadic BC (13, 14). Many microarray studies have led to the discovery of several genes associated with BC. However, most gene expression profiling studies have been carried-out in Caucasian populations, and while study of non-Caucasian populations has been minimal (15). Significant risk factors of BC in the western countries such as nulliparity, low parity, first time pregnancy at late age, no history of breast feeding etc., are usually not common in the Saudi society, yet BC incidence is still high among women from the Kingdom of Saudi Arabia (16).

A comprehensive molecular and genomic analysis of breast tumors consists of the study of choice to understand the complexity and severity of the disease and to find biomarkers as 'druggable' molecular targets. Transcriptomic analysis coupled to functional and pathway analysis can lead to new insight into biomarkers and signatures associated with the disease. De-regulated signaling pathways are thought to drive functional processes such as cell growth, cellular proliferation, and invasion of cancer cells. Thus, identifying such underlying driving events will be vital for studies of tumor progression and for the identification of novel therapeutic targets.

Matrix metalloproteinases (MMPs) degrade proteins in the extracellular matrix by their endopeptidase activity and so far 23 MMPs have been reported (17). Based on their substrate specificity, MMPS are further classified into collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and -26), enamelysin (MMP-20), membrane-bound MMPs (MMP-14 to -17, MMP-24 and -25) and others (MMP- 19, -21, -23, -27 and -28) (8). Matrix metalloproteinases (MMPs) are up-regulated in almost every type of cancer and their expression is linked to a variety of vital aspects of cancer progression including proliferation, invasion, epithelial-to-mesenchymal transformation, metastasis and angiogenesis (18). MMPs are often associated with a poor prognosis for patients (19).

Several studies have described the presence and role of MMPs in many types of cancers including BC (18, 20). MMPs (MMP-2, -7, -9, -10, -11, -13, -14 and -15) have been found to be involved in BC progression and metastasis (21-25). Specifically MMP-1, -2, -3, -7, -9 and -14 are implicated as key factors in tumor invasion, metastasis and angiogenesis (22-25). MMP- 1, and -9 have been linked to cancer cell proliferation, tumor invasion, and epithelial-to-mesenchymal transformation (24, 26-29). A higher concentration of MMP-1 and -9 proteins was detected in BC tissue compared to normal breast tissue by ELISA (30). Studies performed so far have described the presence and role of MMPs in BC, however the role of all MMPs in different key aspects of BC progression is not yet fully-explored. This study was conducted in order to evaluate the prognostic value of MMP-9 in IDC.

Materials and Methods

Patients and samples. The study was performed on female BC patients from the KSA diagnosed with IDC of breast. The samples were collected from either of two participating Medical Centers in Jeddah, including the King Abdulaziz University Hospital, and Bakshs Hospital, during years 2008-2011 for fresh samples and 2000-2011 for FFPE samples. For gene expression analysis, fresh tumor tissue specimens were obtained from fresh surgical resections adjacent to the sites on which final histological diagnosis was performed. Fresh normal breast specimens were derived from surgically-resected normal breast tissues. Samples from 38 tumors (4 G1, 17 G2 and 17 G3) were available for transcriptomic analysis. All collected tissue specimens were immediately placed in RNALater (Invitrogen - Life Technologies, Grand Island, NY, USA) or RPMI 1640 medium (GIBCO-BRL, Grand Island, NY,USA). Clinicopathological features such as age, tumor grade, tumor size, hormone receptor status, lymph node involvement and pathology reports were retrieved from the patients' records after obtaining all the relevant ethical approvals. Histoclinical characteristics of the BC patients are summarized in Table I. Immunohistochemical expression of MMP-9 was validated on 118 cases and evaluation was complemented with corresponding follow-up data. The mean survival time within the follow-up period was of 52.1 months with a range between 1-119 months. The samples' outcomes were defined as overall survival (OS) and disease-free survival (DFS).

All patients included in the study provided written informed consent. The study was reviewed and approved by the Center of Excellence in Genomic Medicine Research (CEGMR) ethical committee (approval number 08-CEGMR-02-ETH).

Table I. *Histoclinical characteristics of the 45 breast cancer patients.*

Characteristics	n	%
Gender		
Female only	45	100
Age		
≤50 Years	32	71
≥50 Years	13	29
Age, mean (range) years	48 (27-80)	
Tumor size: mean (sd) (cm)	3.1 (±1.4)	
Site		
Right	25	56
Left	18	40
NA	2	4
Histological type		
IDC	38	85
ILC	3	7
Mixed and others	2	4
NA	2	4
Grade		
1	4	9
2	17	37.5
3	17	37.5
NA	7	16
pN		
Negative	19	42
Positive	23	51
NA	3	7
LI		
Negative	17	38
Positive	19	42
NA	9	20
ER (IHC)		
Negative	19	42
Positive	19	42
NA	7	16
PR (IHC)		
Negative	27	60
Positive	11	24
NA	7	16
HER2 (IHC)		
Negative	24	53
Positive	14	31
NA	7	16
TNRS		
Yes	11	24
No	34	76

N: Number of cases; NA: information not available; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; pN: pathological lymph node involvement; LI: lymphatic invasion; ER: estrogen receptor; IHC: immunohistochemistry; PR: progesterone receptor; TNRS: triple-negative receptor status.

RNA extraction and array processing. Total RNA was extracted from fresh breast tissue specimens with the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) including an on-column DNAse treatment according to manufacturer's recommendations. The quality and quantity of the purified RNA was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Mean value of RNA integrity number (RIN) for all 50 processed samples was 8.0. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Three hundred ng of each RNA sample were processed according to the manufacturer's recommendation (Affymetrix, Santa Clara, CA, USA). After fragmentation and labeling, the samples were hybridized at 45˚C for 17 h to Human Gene 1.0 ST GeneChip arrays (Affymetrix, Santa Clara). These arrays are conceptually based on the Human Genome sequence assembly UCSC hg18, NCBI Build 36 and interrogate with a set of 764,885 probes 28,869 annotated genes.

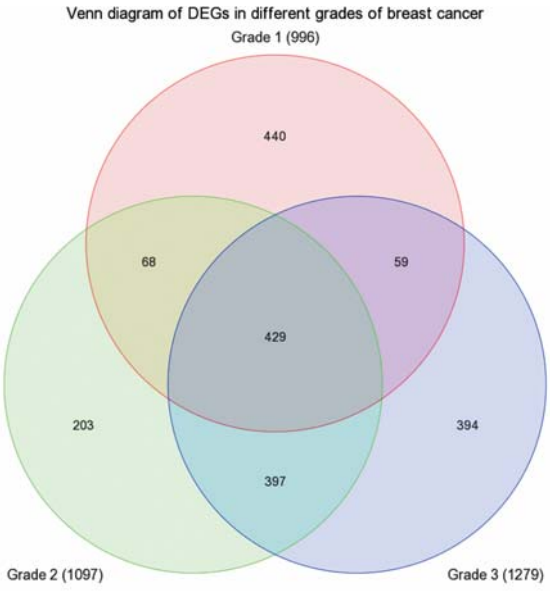

Figure 1. *Venn diagram showing differentially expressed genes in different grades of breast cancer compared to normal controls (996 for G1, 1097 for G2 and 1279 for G3). 429 genes were expressed in all grades whereas 440, 203 and 394 genes were exclusively expressed in G1, G2 and G3 respectively.*

Gene expression analysis. Affymetrix. CEL files and were imported to Partek Genomics Suite version 6.5 (Partek Inc., MO, USA). Data were normalized using RMA normalization. Principal component analysis (PCA) was performed on all probes to visualize for high- dimensional data. PCA was used to assess quality control as well as overall variance in gene expression between disease states. Analysis of Variance (ANOVA) was applied on the complete data set and differentially expressed gene list was then generated using false discovery rate (FDR) of 0.05 with 2-fold change cut-off. Unsupervised two-dimensional average linkage hierarchical clustering was performed using Spearman's correlation as a similarity matrix. The microarray data generated in this study are in compliance with MIAME (http://www.mged.org/Workgroups/ MIAME/miame.html) guidelines. The complete dataset and associated clinicopathological information were submitted to NCBI's Gene Expression Omnibus (GEO) and are accessible through accession number GSE36295.

Immunostaining of MMP-9. Immunohistochemistry was performed in 4-μm thick sections of 118 FFPE samples as a validation step. MMP-9 expression was performed using a rabbit polyclonal anti-

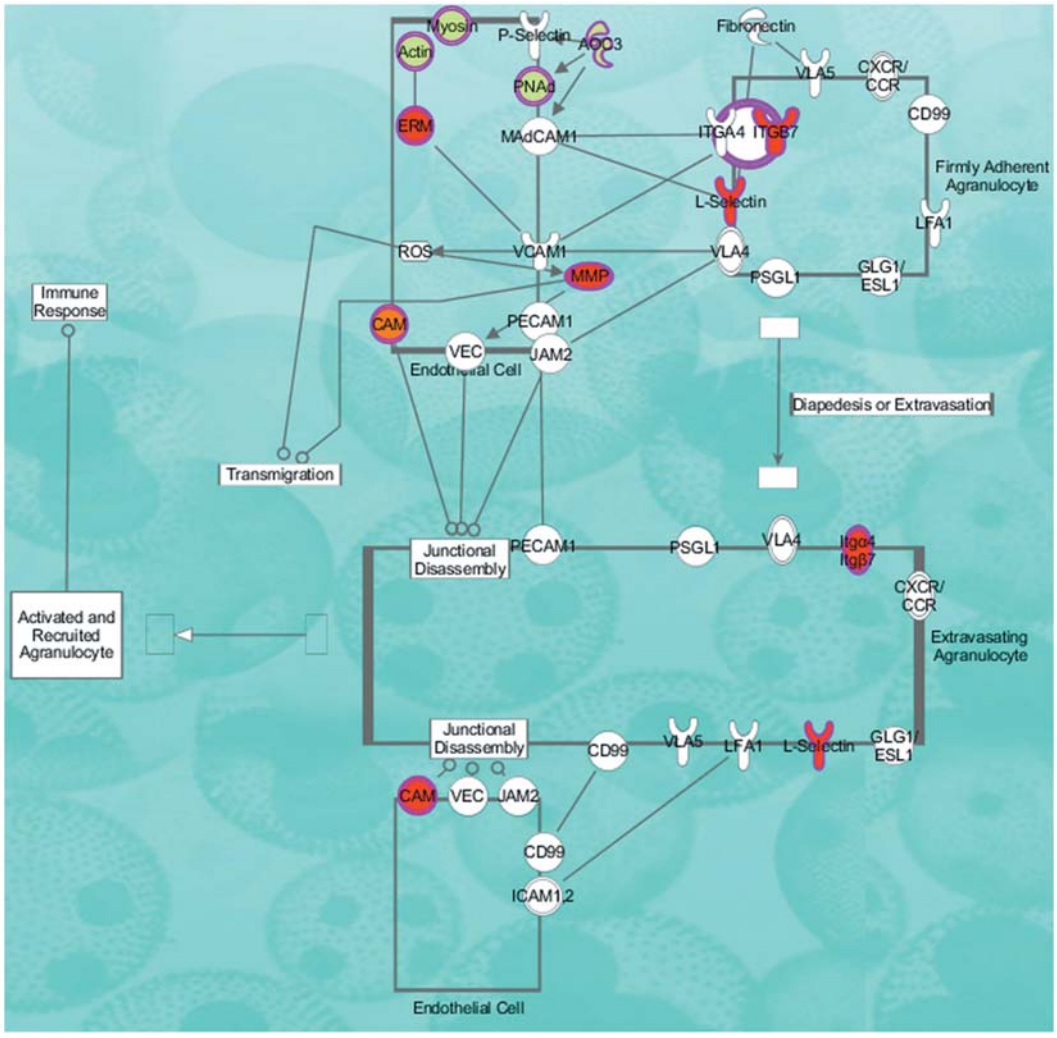

Figure 2. *Agranulocyte adhesion and diapedesis pathway. Transcriptomic signatures of high-grade breast cancer showed a significant disruption in agranulocyte adhesion and diapedesis pathway. MMP-9, -11 and -13 are up-regulated and involved in trans-migration and junctional disassembly. Red color represents overexpression and green color is for down-regulation.*

MMP-9, 0.7 ml ready-to-use from Spring Bioscience (LOT: 110622, USA). Immunostaining was carried out in an automated immunostainer (BenchMark XT, Ventana Medical systems Inc., Tucson, AZ, USA) according to the manufacturer's instructions and interpretation of MMP-9 expression patterns was graded using the index score as mentioned in previous studies (31-32).

Functional and pathway analysis. To define biological networks, interaction and functional analysis among the differentially-regulated genes in BC, pathway analyses were performed using the Ingenuity Pathways Analysis software (IPA) (Ingenuity Systems, Redwood City, CA, USA). Statistically significant differentially expressed datasets containing 996 genes for G1, 1,097 genes for G2, 1,279 genes for G3 and their corresponding probesets ID, Gene symbol, Entrez gene ID as clone identifier, *p*-value and fold change values were uploaded into IPA. The functional/pathway analysis of IPA identifies the biological functions and/or disease and pathways that are most significantly altered for the differentially expressed gene set. The significance of the connection between the expression data and the canonical pathways were calculated by ratio and/or Fisher's exact test.

Biostatistics. Statistical analyses were performed using the IBM SPSS® Statistics (IBM Company, New York, NY, USA) software package (IBM SPSS Statistics for Mac, version 21). Fischer's exact test (2-sided) was used to assess significance of the associations between MMP-9 expression and samples categorical variables. Univariate survival analysis for outcome measures [overall survival (OS), disease-free survival (DFS)] was based on Kaplan-Meier method, with log-rank (Mantel-Cox) comparison test. In all tests, $p<0.05$ was regarded as statistically significant.

Results

The main focus of the present study was to search for novel biomarkers through transcriptomic profiling and immuno-

histochemical analysis in Saudi Arabian women. Based on our transcriptomic profiling, we validated MMP-9 expression in IDC samples (n=118), and determined its prognostic potential.

We profiled 38 fresh breast tissue specimens (4 G1, 17 G2 and 17 G3) and compared them against 8 normal control samples. We performed PCA scatter plot for visualizing the high-dimensional array data where each point represents a chip or sample. We applied PCA for identifying outliers and major effects in the data. The results of PCA of the transcriptomic data showed that the samples from the same tissue type clustered tightly together. Clear differences were also observed between these tumors and normal tissues revealing distinct expression profiles for the different tissue types (Figure 7). PCA mapping showed that 24.5% of the overall variance in the microarray dataset is depicted by the first three principal components.

Identification of differentially expressed genes. Comparison of the genome-wide expression of breast cancer grades revealed 1,593 differentially expressed genes with 2-fold change, false discovery rate $p<0.05$. Transcriptional profiling revealed thousands of genes associated to BC. Cluster analysis also revealed that the cancer tissues agglomerated in various subsets according to grade. We also investigated whether pathway-based clustering is evident in the gene signature for specific grades. For this reason, genes from each signature were extracted and analyzed for significantly-altered biological processes and pathways. Additionally, we identified differentially expressed genes for each grade (996 for G1, 1,097 for G2 and 1,279 for G3 *vs*. Normal). A venn diagram of grade-specific genes reveals that expression of some genes were restricted to fixed grade, however many genes had shown linked expression and some of them were expressed in all grades (Figure 1).

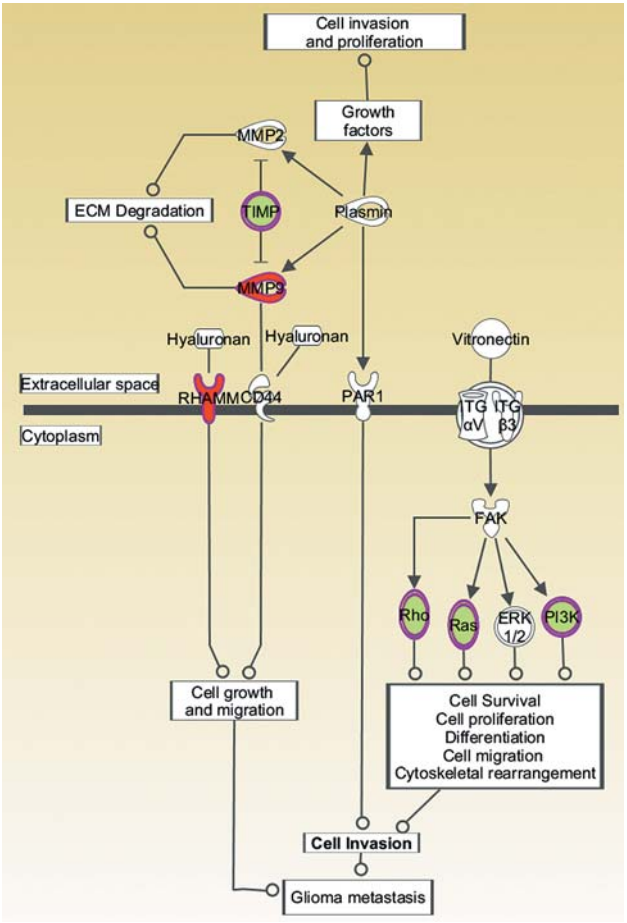

Figure 3. *Glioma invasiveness signaling pathway shows the role of MMP-9 in extracellular matrix degradation, tumor proliferation, invasion and metastasis. TIMP1, a inhibitor MMP-9, is down-regulated. Red color represents over-expression and green color denotes down-regulation.*

Pathways and networks underlying breast cancer. To understand the mechanisms by which the genes alter a wide range of physiological processes, we examined bio-functions, molecular networks and pathways associated with BC and its different grades. Interestingly, the biological process, of cellular movement was significantly over-represented in both down-regulated and up-regulated gene lists pointing that metastasis were linked to a different equilibrium of switching on and off. Functional analysis of BC-associated genes revealed an overexpression of genes involved in cell-cycle progression, DNA repair, cell death, tumor morphology and tissue developments.

Transcriptomic signatures of grade 2 and 3 showed significant disruption in signaling pathways associated genes of the agranulocyte adhesion and diapedesis (Figure 2), ILK signaling, granulocyte adhesion and diapedesis, molecular mechanisms of cancer, glioma invasiveness signaling (Figure 3), FAK signaling, IL-8 signaling and regulation of the epithelial-mesenchymal transition pathway (Table II). Further pathway analysis of differentially-regulated genes provides novel hypotheses underlying tumor invasion and metastatic progression of BC.

MMPs expression and clinical significance of MMP-9. Analysis by IPA shows a set of key genes disrupting a pathway in a way that it results in tumor initiation or progression. Several MMPs (MMP-1, -9, -11, -12 and -13) were significantly over expressed in different grades of BC and involved in many canonical pathways (Table III). As shown in Figure 4, unsupervised hierarchical clustering revealed distinct gene patterns among cancer grades (G1, G2, G3) and normal (G0).

MMP-9 was found to be significantly overexpressed in G2 and G3 and plays an important role in tumor invasion. Additionally, protein level expression of MMP-9 was

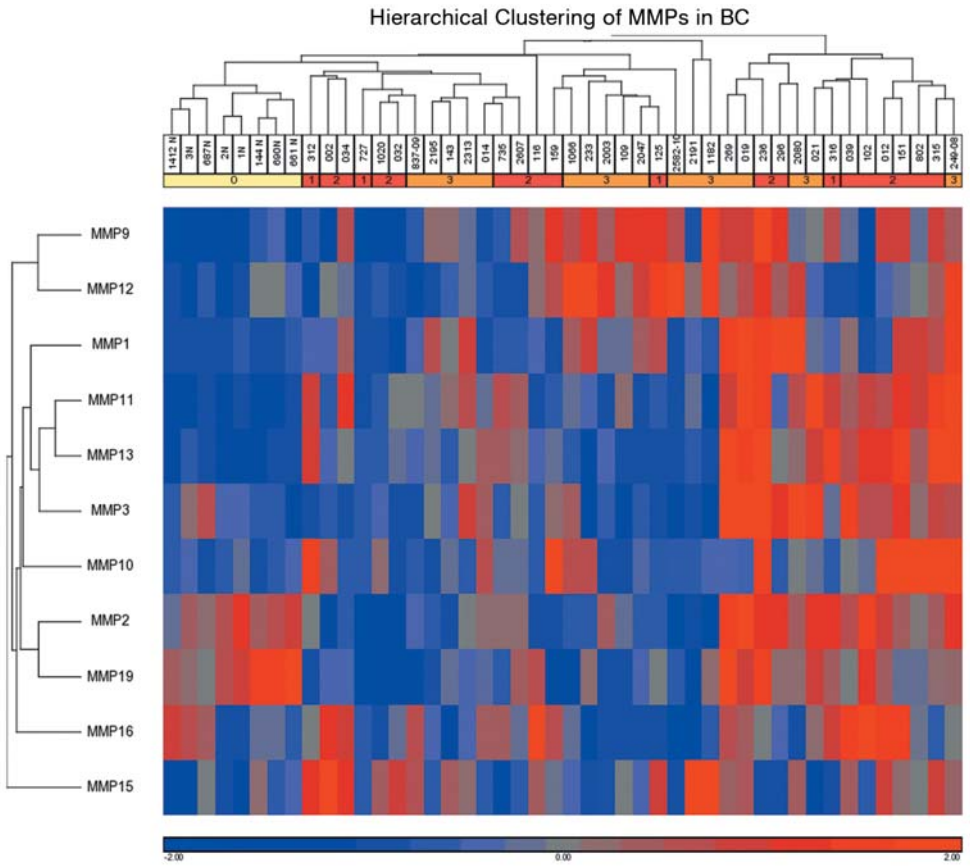

Figure 4. *Hierarchical clustering and Functional analysis of altered MMPs gene expression data of breast cancer using Affymetrix Human ST 1.0 array and Partek GS 6.5 software. The cluster color represents the normalized expression level of a given gene in a particular histopathological grade (0, 1, 2 and 3 represent, normal, grade1, grade 2 and grade 3 respectively).*

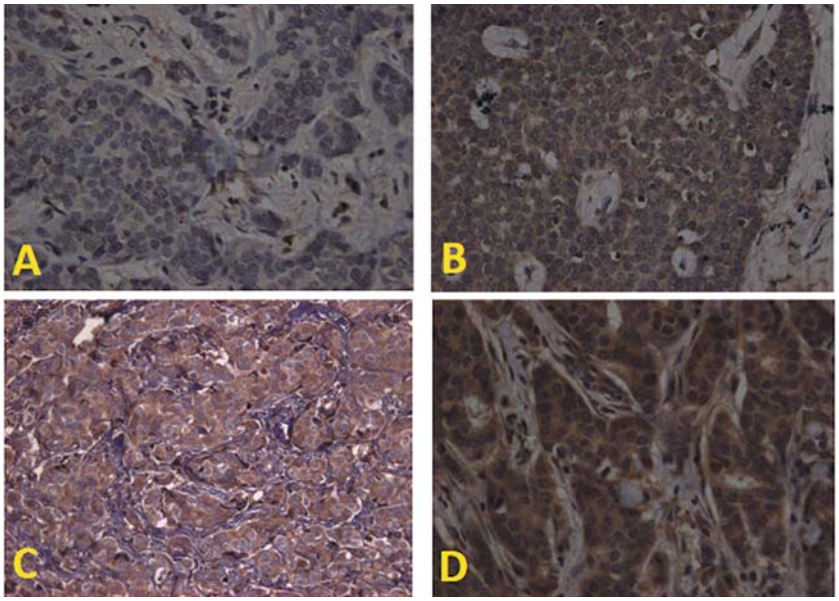

Figure 5. *Immunohistochemical staining of invasive ductal carcinoma of breast tissues showed different MMP-9 expressions. A: no expression, B: weak expression, C: moderate expression and D: strong expression. The expression patterns were pictured using a Coolsnap Pro Color camera and ImagePro® Plus software (Media Cybernetics, Inc., USA). Original magnification, ×40.*

validated on 118 BC samples and found to be significantly high expressed in G2 and G3 (p=0.0313) (Table IV). The pattern of MMP-9 expression in IDC samples is presented in Figure 5. The level of expression (the staining index (I)) in each sample was calculated using the following formula; I=0 × f0+1 × f1 +2 × f2 +3 xf3. The obtained results were varied between 0 (negative), 1 (low), 2 (moderate) and 3 (high). The mean MMP9 expression level in the IDC samples was 1.6. The pattern of MMP-9 expression in IDC samples was categorized into no/weak expression which was noticed in 45% of cases and into moderate/strong expression which was observed in 55% of the cases. According to histological grade; 22 samples were G1 (18.6%), 61 were G2 (50.8%), and 35 were G3 (29.66%). Twenty five of 118 (20.8%) IDC cases had recurrence events and 73 of 118 (60.8%) cases were recurrence-free. At the end of the follow-up period, 84 of 118 patients (70%) were recorded alive, and 22 (18.3%) were recorded as deceased. Immunohistochemical staining of MMP-9 did not significantly correlated with survival of patients (p=0.08 for overall survival and p=0.7 for disease-free survival) (Figure 6).

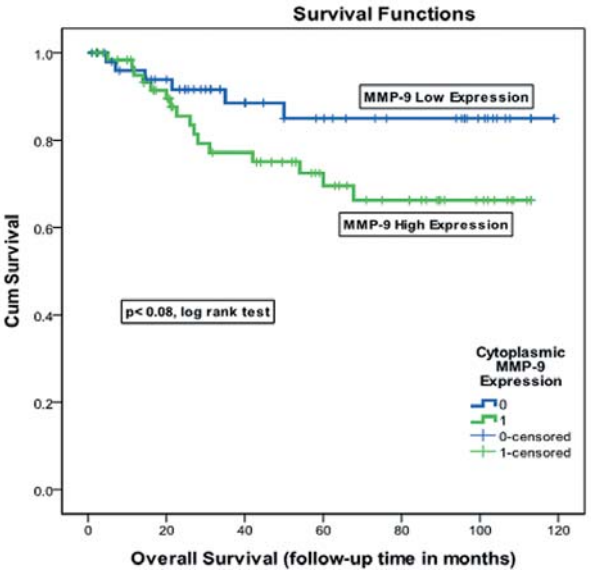

Figure 6. *Kaplan-Meier overall survival curve according to the MMP-9 expression. High expression of MMP-9 is distinctly associated with overall survival (p=0.08).*

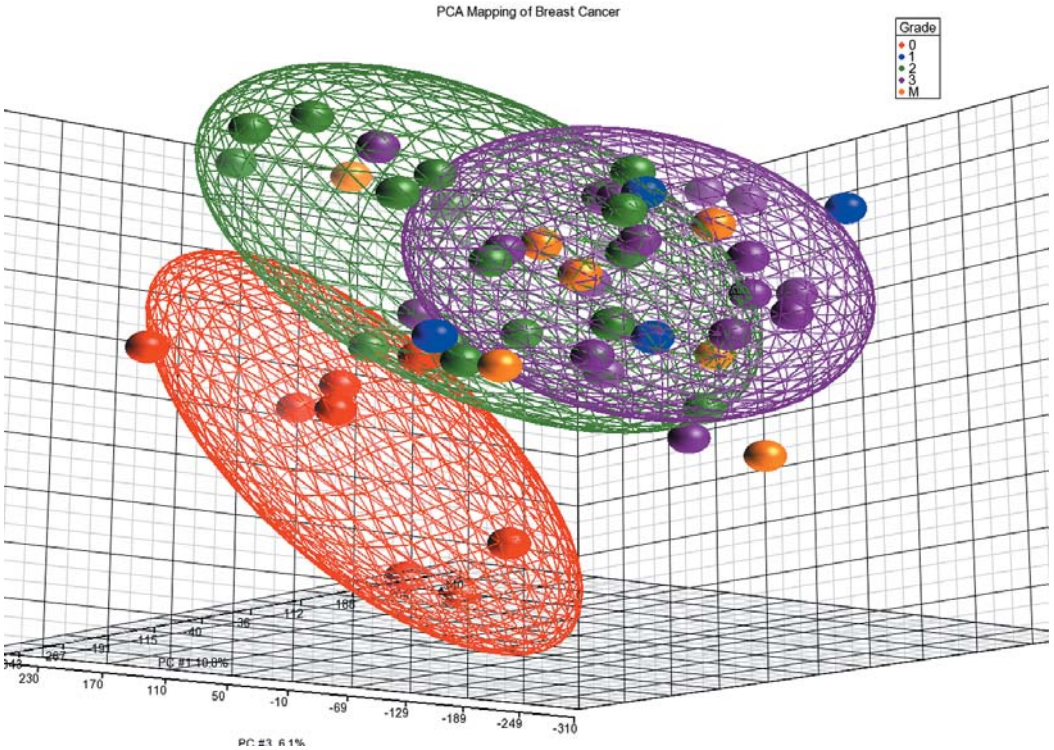

Figure 7. *Principal component analysis of transcriptomic data set. The top three principal components are plotted on the X-, Y-, and Z-axes, respectively. Overall variation between cancer and normal sets, where each spot represents an individual array, can be seen by the clustering within each tissue type and the separation between the different tissue types. Green, Pink and Red eclipses represent grade 2, grade 3 and normal respectively.*

Table II. *Canonical pathways predicted by the Ingenuity Pathway Analysis software for Grade 2 and 3.*

Ingenuity canonical pathways	-log(*p*-value)	Ratio	Molecules
Agranulocyte adhesion and diapedesis	4.81E00	1.36E-01	AOC3,CLDN10,CLDN11,CXCL9,GNAI1,CXCL12,MMP13,IL1R1,CLDN7, IL33,CXCL10,CCL13,CLDN4,CLDN8,EZR,CCL28,PECAM1, MMP11,ACTG2,CXCL2,CX3CL1,CD34,MMP-9,MYH1
ILK signaling	5.95E00	1.33E-01	FLNB,PIK3R1,BMP2,RHOH,CFL2,PPAP2B,RHOU,FIGF,AKT3,ACTG2, MYH1,MUC1,FERMT2,PIK3C2G,VIM,RHOJ,CREB5,CDH1,RHOQ, RND3,FLNC,ARHGEF6,MAPK10,LEF1,KRT18,PPP2R1B,MMP9
Granulocyte adhesion and diapedesis	4.23E00	1.28E-01	CLDN10,CLDN11,CXCL9,GNAI1,CXCL12,MMP-13,IL1R1, CLDN7,IL33,CXCL10,CCL13,CLDN8,CLDN4,EZR,CCL28, PECAM1,MMP11,TNFRSF1B,CXCL2,CX3CL1,MMP-9,HSPB1
Molecular mechanisms of cancer	4.21E00	9.47E-02	FYN,PIK3R1,BMP2,PSENEN,MAPK13,RHOH,CHEK1,TGFBR2,BMP8A, FANCD2,ADCY5,MRAS,RHOU,E2F5,AKT3,PTCH2,CDC25C,CCNE2, STK36,PIK3C2G,GNAI1,RHOJ,AURKA,BAK1,CDH1,PRKAR2B,CCND2, RHOQ,FZD4,RND3,PAK3,ARHGEF6,MAPK10,LEF1,BMP6,GNAL
Axonal guidance signaling	4.13E00	8.82E-02	SLIT3,DPYSL2,FYN,PLXNA3,RND1,PIK3R1,BMP2,UNC5B,SEMA6A, CXCL12,MMP13,ABLIM1,GNG11,BMP8A,SEMA3D,IGF1,CFL2,ABLIM3, MRAS,FIGF,AKT3,TUBA1C,ERBB2,ADAMTS5,PTCH2,SEMA3G, STK36,ADAMTS1,WNT2B,GNG2,GNAI1,PIK3C2G,SLIT2,PLCL2, PRKAR2B,FZD4,PAK3,ARHGEF6,PDGFD,BMP6,MMP-9,GNAL
Glioma invasiveness signaling	3.73E00	1.69E-01	TIMP4,RHOQ,RND3,HMMR,PIK3R1,MRAS, RHOU,PIK3C2G,RHOJ,RHOH,MMP-9
FAK signaling	3.67E00	1.33E-01	FYN,CAPN6,ARHGAP26,HMMR,PIK3R1,PIK3C2G, TLN2,PAK3,ARHGEF6,MRAS,AKT3,ACTG2,TNS1,EGFR
ATM signaling	3.66E00	1.69E-01	RAD51,CDC25C,FANCD2,MAPK10,CCNB2, MAPK13,CREB5,BLM,CDK1,CHEK1,CCNB1
Colorectal cancer metastasis signaling	3.5E00	1.01E-01	PIK3R1,MMP13,RHOH,TGFBR2,GNG11,ADCY5,RHOU,MRAS,FIGF, AKT3,MMP11,EGFR,ADRBK2,WNT2B,GNG2,PIK3C2G,RHOJ,CDH1, PRKAR2B,RHOQ,FZD4,RND3,MAPK10,LEF1,TCF7L2,MMP-9
Leukocyte extravasation signaling	3.49E00	1.12E-01	CLDN10,CLDN11,ARHGAP6,PIK3R1,JAM2,PIK3C2G,GNAI1,CXCL12, MMP13,BMX,MAPK13,CLDN7,RHOH,TIMP4,CLDN4,CLDN8, EZR,MAPK10,PECAM1,MMP11,ACTG2,DLC1,MMP-9
Atherosclerosis signaling	3.23E00	1.18E-01	PLA2G16,CMA1,CD36,CXCL12,MMP13,F3,PLA2G2A,IL33, PLA2G4A,ALB,LPL,COL10A1,PDGFD,MMP-9,APOD,RBP4
IL-8 signaling	2.7E00	9.05E-02	ANGPT1,PIK3R1,GNG2,PIK3C2G,GNAI1,RHOJ,IRAK3,RHOH,CDH1, RHOQ,GNG11,CCND2,RND3,MAPK10,MRAS,RHOU,AKT3,FIGF,MMP-9,EGFR
Human embryonic stem cell pluripotency	2.65E00	1.01E-01	FGF2,BMP2,PIK3R1,WNT2B,PIK3C2G,INHBA,TGFBR2, FZD4,BMP8A,MRAS,S1PR1,AKT3,LEF1,BMP6,PDGFD,TCF7L2
Inhibition of angiogenesis by TSP1	2.56E00	1.75E-01	TGFBR2,FYN,MAPK10,CD36,AKT3,MAPK13,MMP-9
Regulation of the epithelial-mesenchymal transition pathway	2.55E00	9.79E-02	ESRP2,FGF2,PIK3R1,WNT2B,PIK3C2G,PARD6B, PSENEN,TGFBR2,CDH1,FZD4,HGF,MRAS,AKT3, LEF1,PDGFD,FGF7,MMP-9,TCF7L2,EGFR
Germ cell-sertoli cell junction signaling	2.43E00	1.02E-01	PIK3R1,PIK3C2G,RHOJ,GSN,RHOH,TGFBR2,CDH1,RHOQ,PAK3, RND3,SORBS1,PPAP2B,RHOU,MAPK10,MRAS,TUBA1C,ACTG2

The table shows the significantly over-represented canonical pathways across the whole dataset of differentially expressed genes.

Table III. *Microarray based expression of MMPs in breast cancer grades 1, 2 and 3.*

Gene symbol	Gene name	RefSeq	FC (G1 *vs*. Control)	FC (G2 *vs*. Control)	FC (G3 *vs*. Control)
MMP-1	Matrix metallopeptidase 1 (interstitial collagenase	NM_002421	2.17, NS	3.21	5.26
MMP-9	Matrix metallopeptidase 9 (gelatinase B, 92kDa)	NM_004994	2.38, NS	3.68	4.47
MMP-11	Matrix metallopeptidase 11 (stromelysin 3)	NM_005940	3.01	4.25	3.66
MMP-12	Matrix metallopeptidase 12 (macrophage elastase)	NM_002426	1.45, NS	1.88, NS	3.55
MMP-13	Matrix metallopeptidase 13 (collagenase 3)	NM_002427	4.96	5.79	3.61

p-Value; significant if $p<0.05$. FC: Fold change, significant if FC>2. NS: Non-significant.

Discussion

Breast cancer is an epithelial tumor with high invasive and metastatic potential. Poorly differentiated tumors (G3) and moderately differentiated tumors (G2) seem to have a higher invasive potential resulting in a higher frequency of lymph node metastases and lymphangiosis carcinomatosa than well differentiated tumours (G1) (33). Many BC biomarkers for early detection, monitoring of advanced BC, prognosis, therapeutic prediction, and monitoring of patients' outcomes have been proposed (34-35). Unfortunately, most of the biomarkers fail in clinical applications. Human epidermal growth factor receptor 2 (HER2), Estrogen receptor (ER) and Progesterone receptor (PR) are the only well-established biomarkers, currently used for clinical treatment decisions (36). Therefore, emphasis has to attributed to biomarkers research aiming to improve BC therapy.

In the present study, we identified hundreds of significant genes differentially expressed in BC from Saudi Arabia associated with clinical and histological parameters. Many of these genes were associated with cell-cycle regulation and DNA integrity checkpoint networks. A similar transcriptomic signature for many of these genes has been reported for human breast cancer in the past (12, 37-41). The scope of the present study was to focus on biomarkers associated with higher grades, G2 or G3, and their possible roles in tumor invasion and metastasis. Unsupervised clustering analysis also revealed the gene expression signatures to be associated with histological grade. Genes that are expressed preferentially in G1 but not in G2 or G3, may function as biomarkers for cancer initiation; whereas genes expressed in G2 or G3 but not in G1, could be novel biomarkers for tumor invasion and metastasis.

Several studies have shown that overall expression of number of MMPs increases along with breast tumor progression and some MMPs correlate with tumor invasion, metastasis or poorer outcome (42-43). Our results showing overexpression of MMP-1, -9, -11, -12 and -13 in tumor in comparison to normal breast tissue coincides with findings from other studies. MMP-9 is a potential biomarker, and its high expression has been documented in many cancer types (44), however its expression is not consistent in all studies conducted on BC. Elevated MMP-9 expression in IDC has been shown to be a potential biomarker in some studies (43, 45). Conversely, other studies revealed different results and recommended further investigations (45, 46).

Table IV. *The correlation between the MMP-9 expression between histological grades of breast cancer.*

Characteristics	Patients, N (%) with MMP-9 expression		*p*-Value
	Negative (0, 1)	Positive (2, 3)	
Histological grade			
Grade 1	15 (62)	7 (38)	0.0313
Grade 2 + 3	39 (44)	57 (56)	

MMP-9 was expressed in most of the IDC samples (97.5%) and elevated in 55% of the IDC samples. Additionally, MMP9 expression had a border line correlation with the samples overall survival (OS). Other studies have found MMP-9 expression by IHC in 52-62% of the cases (47-49). We identified a very significant correlation between tumor grade and MMP-9 expression. Similarly to our finding, other studies also indicated significant correlation between the expression of MMP-9 and tumor grade (49-50). On the other hand, many studies did not find significant correlation between the expression of MMP-9 and tumor grade (18, 48, 51-52). Tumor grade and tumor stage are well-established prognostic markers, therefore, MMP-9 carries potential prognostic value. However, we did not find any significant correlation of MMP-9, neither with DFS nor with OS. From the Kaplan-Meier OS plot it can be deviated that there is a constant difference between the curve of MMP-9 positive and the negative samples after the follow-up period of 60 months. Some studies had found significant correlations of MMP-9 expression with the OS and DFS in IDC patients (42, 45, 46) while other studies had not detected any correlations (48, 51). Thus, further investigations have to be done to assess the prognostic

importance of MMP-9 in IDC. Currently, the routine BC prognostic factors are tumor size, lymph node status, histological grade and type (42). However, these prognostic factors have found their limitations in many BC cases (53). Therefore, adding a new validated prognostic biomarker, such as a matrix metalloproteinase to the existing prognostic factors would be extremely valuable in guiding BC patients' treatment and enhancing their survival chances (42).

Conclusion

In the present study, combined transcription profiling and pathway analysis revealed that MMPs (MMP-1, -9, -11, -12, and 13) are differentially-regulated in different BC grades and might play important roles in tumor invasion and metastasis. Additionally, we found a high expression of MMP-9 in IDC, with significant association to grade, yet a limited prognostic value. Thus, MMPs in general and MMP-9 in particular, are of immense value and warrant further study as diagnostic markers and potential drug targets.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

Acknowledgements

This work was supported by King Abdullah City for Science and Technology, Riyadh, Saudi Arabia (KACST, Strategic Project ID: 10-BIO1073-03 and 10-BIO1258-03) and Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah, Saudi Arabia (CEGMR Project ID: CEGMR-N08-14). The authors would like to thank Nuha Alansari, Alaa Alboogmi, Manal Shaabad, Manar Ata and Nada Salem for performing RNA extraction, bioanalyzer assays, microarray experiments, immunostaining and statistical analysis. We thank the patients, and the contributions of physicians, nurses, and pathologists of the King Abdulaziz University Hospital and Bakshs Hospital, Jeddah, the Kingdom of Saudi Arabia.

References

1 Karimi P, Shahrokni A and Moradi S: Evidence for U.S. Preventive Services Task Force (USPSTF) Recommendations Against Routine Mammography for Females between 40-49 Years of Age. Asian Pac J Cancer Prev *14(3)*: 2137-2139, 2013.

2 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61(2)*: 69-90, 2011.

3 Youlden DR, Cramb SM, Dunn NA, Muller JM, Pyke CM and Baade PD: The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality. Cancer Epidemiol *36(3)*: 237-248, 2012.

4 American Cancer Society (ACS). Global Cancer Facts and Figures 2012. Atlanta, GA: American Cancer Society; 2007. Available from: http://www.cancer.org/acs/groups/content/@nho/documents/document/globalfactsandfigures2007rev2p.pdf. Accessed December 9, 2013.

5 Saudi Cancer Registry, Cancer Incidence Report 2009. Saudi Arabia. www.scr.org.sa December 2012.

6 Chen, Y. Breast Cancer. http://www.nlm.nih.gov/medlineplus/ency/article/000913.htm 2012.

7 Verkooijen HM, Fioretta G, Vlastos G, Morabia A, Schubert H, Sappino AP and Bouchardy C: Important increase of invasive lobular breast cancer incidence in Geneva, Switzerland. International Journal of Cancer *104(6)*: 778-781, 2003.

8 Nagase H, Visse R and Murphy G: Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res *69(3)*: 562-573, 2006.

9 Page-McCaw A, Ewald AJ and Werb Z: Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol *8*: 211-233, 2007.

10 Stamenkovic I: Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol *10*: 415-433, 2000.

11 National Cancer Institute, Breast Ductal Carcinoma. http://cancergenome.nih.gov/cancersselected/breastductal, 2011.

12 Kumar R, Sharma A and Tiwari RK: Application of microarray in breast cancer: An overview J Pharm Bioallied Sci *4(1)*: 21-26, 2012.

13 Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou MC, Lonning PE, Brown PO, Dale ALB and Botstein D: Repeated observation of breast tumor subtypes in independent gene expression data sets. Proceedings of the National Academy of Sciences, USA *100*: 8418-8423, 2003.

14 Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Elledge R, Gutierrez MC, Elledge R, Mohsin S, Osborne CK, Chamness GC, Allred DC and O'Connell P: Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet *362*: 362-369, 2003.

15 Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y and Pietenpol JA: Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. The Journal of clinical investigation *121*: 2750-2767, 2011.

16 Ezzat AA, Ibrahim EM, Raja MA, Al-Sobhi S, Rostom A and Stuart RK: Locally advanced breast cancer in Saudi Arabia: high frequency of stage III in a young population. Med Oncol *16*: 95-103, 1999.

17 Egeblad M and Werb Z: New functions for the matrix metalloproteinases in cancer progression. Nature Reviews Cancer *2(3)*: 161-174, 2002.

18 Bensona CS, Babua SD, Radhakrishnab S, Selvamuruganc N and Sankara BR: Expression of matrix metalloproteinases in human breast cancer tissues. Disease Markers *34*: 395-405, 2013.

19 Curran S, Dundas SR, Buxton J, Leeman MF, Ramsay R and Murray GI: Matrix metalloproteinase/tissue inhibitors of matrix metalloproteinase phenotype identifies poor prognosis colorectal cancers. Clin Cancer Res *10*: 8229-8234, 2004.

20 Kohrmann A, Kammerer U, Kapp M, Dietl J and Anacker J: Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer *9*: 188, 2009.

21 Eiseler T, Doppler H, Yan IK, Goodison S and Storz P: Protein kinase D1 regulates matrix metalloproteinase expression and inhibits breast cancer cell invasion. Breast Cancer Research *11(1)*: R13, 2009.

22 Mark D and Sternlicht GB. Matrix metalloproteinases as emerging targets in anticancer therapy: Status and prospects. Emerging Therapeutic Targets *4*: 609-633, 2000.
23 Pivetta E, Scapolan M, Pecolo M, Wassermann B, Abu-Rumeileh I, Balestreri L, Borsatti E, Tripodo C, Colombatti A and Spessotto P: MMP-13 stimulates osteoclast differentiation and activation in tumour breast bone metastases. Breast Cancer Research *13(5)*: R105, 2011.
24 Chabottaux V, Sounni NE, Pennington CJ, English WR, van den Brule F, Blacher S, Gilles C,Munaut C, Maquoi E, Lopez-Otin C, Murphy G, Edwards DR, Foidart JM and Noel A: Membrane-type 4 matrix metalloproteinase promotes breast cancer growth and metastases. Cancer Research *66(10)*: 5165-5172, 2006.
25 Surowiak P, Dziegiel P, Zabel M, Matkowski R and Kornafel J: Analysis of estrogen receptor (ER) and estrogendependent pS2 protein expression in cells of mammary ductal carcinoma. Folia histochemica et cytobiologica/Polish Academy of Sciences, Polish Histochemical and Cytochemical Society *39(2)*: 141-142, 2001.
26 Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC and Chambon P: A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature *348(6303)*: 699-704, 1990.
27 Delassus GS, Cho H, Park J and Eliceiri GL: New pathway links from cancer-progression determinants to gene expression of matrix metalloproteinases in breast cancer cells. Journal of Cellular Physiology *217(3)*: 739-744, 2008.
28 Hegedus L, Cho H, Xie X and Eliceiri GL: Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. Journal of Cellular Physiology *216(2)*: 480-485, 2008.
29 Balduyck M, Zerimech F, Gouyer V, Lemaire R, Hemon B, Grard G, Thiebaut C, Lemaire V, Dacquembronne E, Duhem T, Lebrun A, Dejonghe MJ and Huet G. Specific expression of matrix metalloproteinases 1, 3, 9 and 13 associated with invasiveness of breast cancer cells *in vitro*. Clinical and Experimental Metastasis *18(2)*: 171-178, 2000.
30 Przybylowska K, Kluczna A, Zadrozny M, Krawczyk T, Kulig A, Rykala J, Kolacinska A, Morawiec Z, Drezewoski J and Blasiak J: Polymorphisms of the promoter regions of matrix metalloproteinases genes MMP-1 and MMP-9 in breast cancer. Breast Cancer Res Treat *95*: 65-72, 2006.
31 Elzagheid A, Emaetig F, Alkikhia L, Buhmeida A, Syrjänen K, El-Faitori O, Latto M, Collan Y and Pyrhönen S: High cyclooxygenase-2 expression is associated with advanced stages in colorectal cancer. Anticancer Research *33(8)*: 3137-3143, 2013.
32 Buhmeida A, Dallol A, Merdad A, Al-Maghrabi J, Gari MA, Abu-Elmagd MM, Chaudhary AG, Abuzenadah AM, Nedjadi T, Ermiah E, Al-Thubaity F and Al-Qahtani MH: High fibroblast growth factor 19 (FGF19) expression predicts worse prognosis in invasive ductal carcinoma of breast. Tumour Biol. [Epub ahead of print] 2013.
33 Saez RA, McGuire WL and Clark GM: Progonstic factors in breast cancer. Semin Surg Onco *52*: 5198-5203, 1989.
34 David EM and Evelyn HK: Protein Biomarkers for the Early Detection of Breast Cancer. International Journal of Proteomics. vol. 2011, Article ID 343582, 9 pages, 2011.
35 Kurebayashi J: Biomarkers, tumor markers and prognostic markers in breast cancer. Nihon Rinsho *64(3)*: 461-466, 2006.
36 Weigel MT and Dowsett M: Current and emerging biomarkers in breast cancer: prognosis and prediction. Endocrine Related Cancer *17(4)*: 245-262, 2010.
37 Bieche I, Vacher S, Lallemand F, Tozlu-Kara S, Bennani H, Beuzelin M, Driouch K, Rouleau E, Lerebours F, Ripoche H, Cizeron-Clairac G, Spyratos F and Lidereau R: Expression analysis of mitotic spindle checkpoint genes in breast carcinoma: role of NDC80/HEC1 in early breast tumorigenicity, and a two-gene signature for aneuploidy. Molecular Cancer *10*: 23, 2011.
38 Ueki T, Nishidate T, Park JH, Lin ML, Shimo A, Hirata K, Nakamura Y and Katagiri T: Involvement of elevated expression of multiple cell-cycle regulator, DTL/RAMP (denticleless/RA-regulated nuclear matrix associated protein), in the growth of breast cancer cells. Oncogene *27*: 5672-5683, 2008.
39 Kais Z, Barsky SH, Mathsyaraja H, Zha A, Ransburgh DJ, He G, Pilarski RT, Shapiro CL, Huang K and Parvin JD: KIAA0101 interacts with BRCA1 and regulates centrosome number. Molecular Cancer Research *9(8)*: 1091-1099, 2011.
40 O'Brien SL, Fagan A, Fox EJ, Millikan RC, Culhane AC, Brennan DJ, McCann AH, Hegarty S, Moyna S and Duffy MJ: CENP-F expression is associated with poor prognosis and chromosomal instability in patients with primary breast cancer. International J Cancer *120*: 1434-1443, 2007.
41 Knoop AS, Knudsen H, Balslev E, Rasmussen BB, Overgaard J, Nielsen KV, Schonau A, Gunnarsdóttir K, Olsen KE, Mouridsen H and Ejlertsen B: Retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. Journal of Clinical Oncology *23*: 7483-90, 2005.
42 Song J, Su H, Zhou YY and Guo LL: Prognostic Value of Matrix Metalloproteinase 9 Expression in Breast Cancer Patients: A Meta-analysis. Asian Pacific Journal of Cancer Prevention, *14(3)*: 1615-1621, 2013.
43 Fernández-Guinea O, Alvarez-Cofiño A, Eiró N, González LO, Del Casar JM, Fernandez-Garcia B and Vizoso FJ: Low microvascular density at the tumor center is related to the expression of metalloproteases and their inhibitors and with the occurrence of distant metastasis in breast carcinomas. International Journal of Clinical Oncology *18(4)*: 629-640, 2013.
44 Kim HJ, Park CI,Park BW, Lee HD and Jung WH: Expression of MT-1 MMP, MMP2, MMP-9 and TIMP2 mRNAs in ductal carcinoma *in situ* and invasive ductal carcinoma of the breast. Yonsei Medical Journal *47(3)*: 333-342, 2006.
45 Sullu Y, Demirag GG, Yildirim A, Karagoz F and Kandemir B: Matrix metalloproteinase-2 (MMP-2) and MMP-9 expression in invasive ductal carcinoma of the breast. Pathol Res Pract *207(12)*: 747-753, 2011.
46 Wu ZS, Wu Q, Yang JH, Wang HQ, Ding XD, Yang F and Xu XC: Prognostic significance of MMP-9 and TIMP-1 serum and tissue expression in breast cancer. International Journal of Cancer *122(9)*: 2050-2056, 2008.
47 Rahko E, Jukkola A, Melkko J, Paavo P, Bloigu R, Talvensaari-Mattila A and Turpeenniemi-Hujanen T: Matrix metallo-proteinase-9 (MMP-9) immunoreactive protein has modest prognostic value in locally advanced breast carcinoma patients treated with an adjuvant antiestrogen therapy. Anticancer Research *24(6)*: 4247-4253, 2004.

48 Scorilas A, Karameris A, Arnogiannaki N, Ardavanis A, Bassilopoulos P, Trangas T and Talieri M: Overexpression of matrix-metalloproteinase-9 in human breast cancer: a potential favourable indicator in node-negative patients. Br J Cancer *84(11)*: 1488-1496, 2001.

49 Li HC, Cao DC, Liu Y, Hou YF, Wu J, Lu JS and Shao ZM: Prognostic value of matrix metalloproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma. Breast Cancer Res Treat *88(1)*: 75-85, 2004.

50 McGowan PM and Duffy MJ: Matrix metalloproteinase expression and outcome in patients with breast cancer: analysis of a published database. Ann Oncol *19(9)*: 1566-1572, 2008.

51 Pellikainen JM, Ropponen KM, Kataja VV, Kellokoski JK, Eskelinen MJ and Kosma VM: Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in breast cancer with a special reference to activator protein-2, HER2, and prognosis. Clinical Cancer Research *10(22)*: 7621-7628, 2004.

52 Jobim FC, Schwartsmann G, Xavier NL, Uchoa DM, Saciloto M and Chemello N: Expression of MMP-9 and VEGF in breast cancer: correlation with other prognostic indicators. Rev Bras Ginecol Obstet *30(6)*: 287-293, 2008.

53 Donegan WL: Tumor-related prognostic factors for breast cancer. A Cancer Journal for Clinicians *47(1)*: 28-51, 1997.

Received December 16, 2013
Revised January 9, 2014
Accepted January 13, 2014