Potential Biomarkers through Genome-wide Expression Analysis of Breast Cancer Samples from Malaysian Patients

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ABSTRACT

Breast cancer is a serious health concern and still a leading cause of death among women in the world. To explore the complexity of this cancer, we performed microarray analysis on highly selective cancer and normal breast tissues. The aim of this study was to identify differentially expressed genes between both tissues and to elucidate further molecular pathways involved in breast cancer carcinogenesis. Genome-wide expression profiling was performed on fifteen cancer and five normal breast tissues using the Affymetrix GeneChip® Human Gene 1.0 ST array. Supervised hierarchical cluster analysis using filtering parameters of -1.5 to 1.5 fold-change and p-value with False Discovery Rate < 0.05 revealed 404 up-regulated and 463 down-regulated genes. Pathway analysis revealed the significant genes were involved in cell cycle regulation, DNA repair, Hedgehog pathway, histone phosphorylation, TRRAP/Tip60 chromatin remodelling and apoptosis regulation. Among the top 10 significantly overexpressed genes were *CENPF*, *DTL* and *MK167* and these were related to cell cycle regulation. Among the top 10 significant down-regulated genes, *HOXA5* and *NRG1* were found to be associated with Wnt signalling pathway and ErbB signalling pathway respectively. Aberrations in these genes are likely to promote breast cancer carcinogenesis. Our current findings highlighted the importance of differentially expressed genes in breast cancer and their molecular pathways that linked these genes. Further studies are required to validate our findings using larger sample size.

INTRODUCTION

Breast cancer is the most frequent cancer and a serious health concern among females (1, 2). This cancer accounts for more than 410,000 deaths among females each year (3) and 14% of cancer deaths (1). The incidence is substantially increasing in Asian countries compared to other regions of the world (4). In Malaysia, breast cancer was the most common cancer in females with a total of 31.3% in 2005 and the percentage increased to 33.8% in 2007. Among the three major races in this country, the incidence was reported to be highest in Chinese compared to Malays and Indians (2).

Breast cancer is a heterogeneous and complex disease (5). It is caused by both genetic and epigenetic alterations (6). In the past few years, microarray studies have been applied to improve our understanding on the pathogenesis and heterogeneity of breast cancer. Breast cancer has been classified by gene expression studies into luminal A, luminal B, HER2/*neu* overexpressed, basal-like and normal breast-like tumour subtypes (7). This technology has also been

used to predict patients' prognosis, treatment selection and therapeutic target identification in breast cancer (8). The Oncotype DX assay (Genomic Health, Redwood City, California) is an example of an FDA approved assay that contains 21 significant genes identified from gene expression profiling. These signatures were used to select women who would benefit from the addition of chemotherapy to tamoxifen (9, 10). In addition, the Mammaprint assay (Agendia, Amsterdam, The Netherlands) that contains 70 significant genes has also been developed to predict risk of distant metastasis in breast cancer patients and identify the patients who would benefit from systemic chemotherapy (11, 12). Until today, these two assays have yet to be proven to be sensitive and reliable for all high risk women (13).

Microarray has also been used to identify the differentially expressed genes between preoperative biopsies and postoperative breast tumours in a previous study (14). Several early response stress-related genes such as *FOSB* and *DUSP1* and cancer related genes including

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MAPK, *MALAT1* and *RASD1* were found to be differentially expressed. Another study used whole-genome cDNA-mediated Annealing, Selection, extension and Ligation (DASL) assay to identify differentially expressed genes between FFPE and fresh frozen breast tissues (15). They have suggested that results from FFPE samples should not be directly compared with results from fresh frozen breast tissues. This was because the formalin fixation used in FFPE samples may induce significant gene expression changes. In Malaysia, a microarray study on 43 paired samples revealed 33 significantly expressed genes including *CD24, CD36, CD9, TACSTD1, TACSTD2, HBB, LEP, LPL, AKR1C1, AKR1C2* and *AKR1C3* in breast tumours compared to normal breast tissues (16). However the study used whole tissues that were likely to contain both cancer and non-cancerous tissues and there was no validation step done to confirm their results.

Despite the technological advances in genomics and transcriptomics, genome-wide microarray based expression analysis is still a reliable tool to allow better understanding of the breast cancer. In this study, we used gene expression profiling by microarray to identify the differentially expressed genes and to determine the molecular pathways that linked these genes in macro- and micro-dissected breast cancer tissues.

MATERIALS AND METHODS

CLINICAL SAMPLES

Subjects were recruited from the Universiti Kebangsaan Malaysia Medical Centre (UKMMC) and Hospital Kuala Lumpur (HKL), Malaysia. Fifteen primary breast tumours and five normal breast tissue samples were collected from 14 Malays and six Chinese patients. All subjects volunteered to participate in this study and signed the informed consent forms. Only patients who have not received chemotherapy or hormone therapy were included. All samples were properly collected and placed in liquid nitrogen before being transferred to the biobank at the UKM Molecular Biology Institute (UMBI) for processing and storage. Tissues were stored at -80°C until further analysis.

All tissues were sectioned into 5 to 7 µm thickness using a cryostat (Microtome Cryostat HM 550; MICROM International GmbH, Walldorf, Germany) and stained with Haematoxylin and Eosin. The slides were then evaluated and confirmed by the histopathologist from the Department of Pathology, UKMMC. Only tissues with more than 80% of malignant cells were subjected to total RNA isolation. Normal tissue has to be free from malignant or inflammatory cells. For tissues that contain less than 80% of cancer cells, Veritas LCM system (Arcturus Engineering, Mountain View, CA) was used to capture the isolated cells. The staining and laser capture procedures were carried out as previously described (17).

RNA ISOLATION

Total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) and quantified using the NanoDrop (Thermo Fisher Scientific, UK) and Agilent RNA 6000 Nano Kit (Agilent Technologies GmbH, Waldbronn, Germany). Only samples with optical densities at 260/280 nM wave length within the range of 1.8 to 2.2 and RNA integrity number of more than 6.5 were included in this study.

GENE EXPRESSION PROFILING

Microarray profiling of 15 tumours and five normal breast tissues were performed using the GeneChip® Human Gene 1.0 ST array (Affymetrix Inc., Santa Clara, CA), which contains 28,869 well annotated genes with 764,885 distinct probes. First, cDNA was amplified using the Applause WT-Amp ST system (NuGEN, Technologies, Inc., San Carlos, CA). This step involved the generation of first strand cDNA, generation of DNA/RNA heteroduplex double-stranded cDNA, Single Primer Isothermal Amplification (SPIA) amplification and post-SPIA modification. The amplified ST-cDNA was further purified using QIAGEN® MinElute® Reaction Cleanup Kit (QIAGEN, Hilden, Germany). Only samples with optical densities at 260/280 nM wave length within the range of 1.8-2.0 and concentration more than 250 ng/µl were selected for the microarray. The cDNA samples underwent fragmentation, biotin labelling and hybridisation on the GeneChip® Human Gene 1.0 ST array. The arrays were then subjected to automated washing and staining using the Affymetrix Fluidics Station 400. Finally, the arrays were scanned with GeneChip scanner.

DATA ANALYSIS

The generated microarray data was extracted using the Affymetrix® Genotyping Console™ (Affymetrix Inc., Santa Clara, CA). These data were further analysed using the Partek Genomics Suite 6.6 (Partek Inc., St. Louis, MO, USA). Data normalisation utilising quantile normalisation and robust multi-array analysis (RMA) background correction was carried out. Differentially expressed genes with fold-change of at least 1.5 and p-value with False Discovery Rate < 0.05 were further analysed. Both Partek Genomics Suite 6.6 and Database for Annotation, visualisation and Integrated Discovery (DAVID) (18, 19) were used for the enrichment analysis and the Pathway Studio (Ariadne, USA) (20) was employed for pathway generation.

RESULTS

The genome-wide expression study was carried out on 15 tumour and five normal breast tissues. The epidemiological characteristics of the patients are shown in Table 1. Using the Partek Genomic Suite, Principal Components Analysis (PCA) was performed to determine the distribution of the

samples. Without eliminating the batch effect, the PCA that acts as a quality control step, clustered the tumour and normal cells distinctly (Figure 1). Filtering characteristic of fold-change -1.5 to 1.5 and p-value with False Discovery Rate < 0.05 yielded a total of 867 differentially expressed genes. In addition, supervised hierarchical clustering revealed 404 up-regulated and 463 down-regulated genes (Figure 2). The top 10 up-regulated genes included *CASC5, CENPF, KIF23, DTL, MK167, TPX2, NUF2, KIF4A, NUSAP1* and *BUB1B* whereas the top 10 down-regulated genes were *PAK3, B3GALT1, CX3CL1, EDN3, KCNMB1, HOXA5, NRG1, KLHL13, TSHZ2* and *IL17RD*.

Table 1. The epidemiological characteristics of the patients

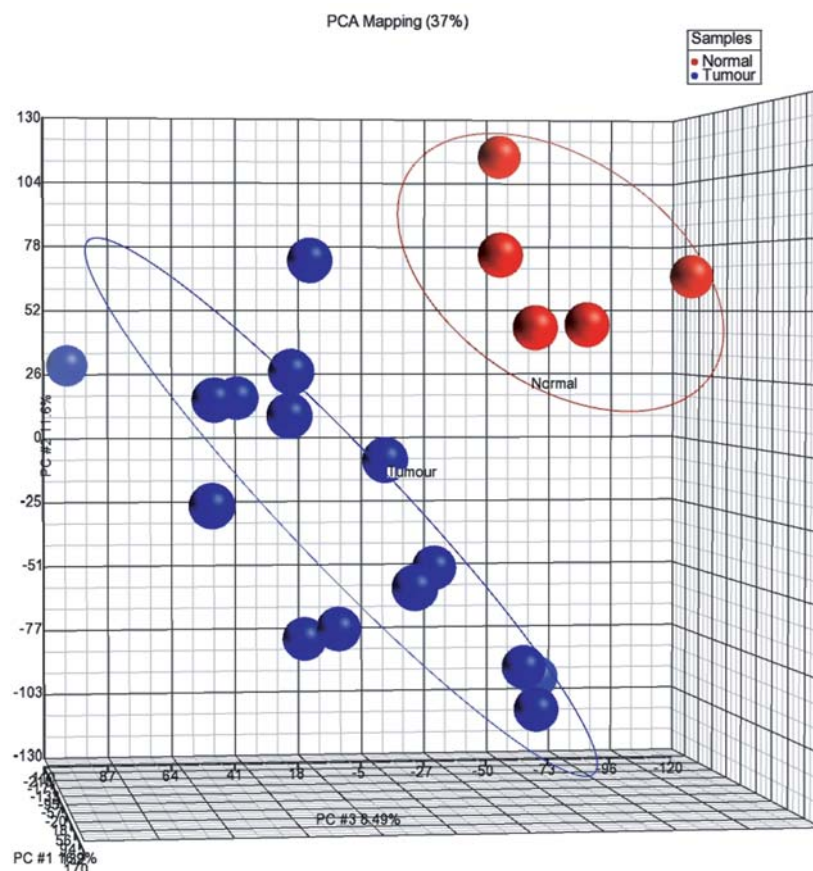|		
Age	Mean	50.6 + 8.24
Range		32-70
Tumour grade	I	15%
	II	40%
	III	45%
Histological type	Infiltrating ductal carcinoma (IDC), not otherwise specified (Nos)	90%
	Non-IDC	10%
Oestrogen Receptor	Positive	75%
	Negative	25%
Progesterone Receptor	Positive	45%
	Negative	55%
Her 2 Amplification	Positive	40%
	Negative	60%
Triple Negative	2 patients	10%

Figure 1. Principle component analysis clustered gene expression profiling of tumour and normal adjacent breast samples. The tumour and normal samples were clustered distinctly. The blue circles indicate tumour samples and the red circles indicate normal samples.
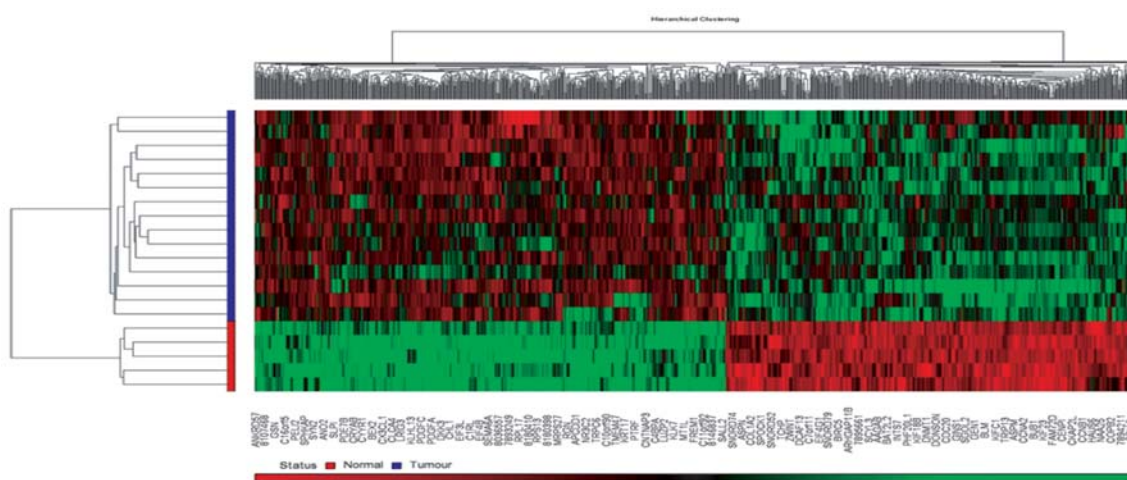
Figure 2. Supervised hierarchical clustering of 15 tumours versus five normal samples display the gene expression intensity for each genes. Samples were clustered based on 785 significant differentially expressed genes at fold change -1.5 to 1.5, p-value with FDR < 0.05. The colour of each small box on the map represents the ratio of gene expression. Green indicates genes were up-regulated above median; red indicates gene were down-regulated below median and black indicates gene were equal to median expression signal. The rows represent individual genes; the columns represent individual sample.

Gene Ontology (GO) is a bioinformatics tool that is used to unify the representation of genes and gene products' attributes across all eukaryotes. It comprises of three aspects that include biological process, molecular function and cellular component (21). GO enrichment analysis on our data under the component for biological process showed that most of the genes were enriched in cell proliferation followed by viral reproduction, pigmentation, growth, rhythmic process, cell killing and metabolic process. Meanwhile, for the molecular function, most of the genes were enriched in chemoattractant activity, structural molecule activity, translation regular activity, enzyme regulator activity, electron carrier activity, catalytic activity, transcription regulator activity, transporter activity and binding activity. For the cellular component, most of the genes were active in extracellular region and synapse (Figure 3).

Enrichment analysis using DAVID bioinformatics tool with filtering characteristic enrichment score of higher than 3, p-value < 0.05 generated a total of 74 genes that were clustered together and highly associated with breast cancer (18, 19). Pathway analysis revealed most of these genes were involved in cell cycle regulation, DNA repair, Hedgehog pathway, histone phosphorylation, TRRAP/ Tip60 chromatin remodelling and apoptosis regulation (Table 2). Interestingly, gene *BLM* was involved in all the pathways as shown in Table 2. In addition, six genes including *FANCD2, MSH2, NBN, RAD54B, TOP2A* and *TOPBP1* were involved in several pathways that were associated with DNA repair such as single-strand base excision DNA repair, single-strand mismatch DNA repair, direct DNA repair and double strand DNA non-homologous repair.
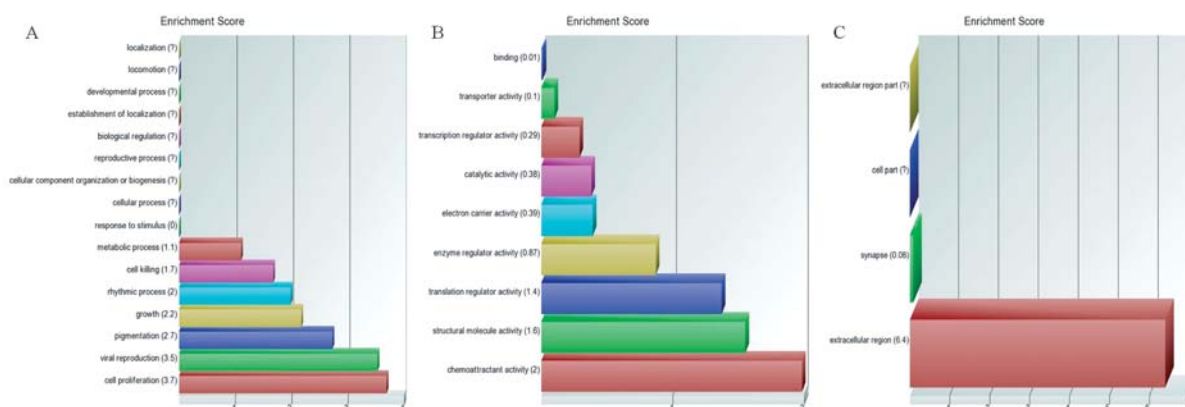

Figure 3. Gene ontology enrichment analysis of 785 differentially expressed genes revealed the enriched biological process, molecular function and cellular component. The number represents the enrichment score and the symbol of ''?' represents zero enrichment score. The high enrichment score means that the genes were found more frequently in the particular ontology whereas zero enrichment score means the genes did not belong to the particular ontology.

Table 2. Pathway analysis with p < 0.05 using Pathway Studio software

Pathway	Genes	p-value
Cell Cycle Regulation	CDK1, DUSP1, CHEK1, E2F1, INHBA, EEF2, MKI67, BRCA1, KIT, FGF1, CCNA2, CCNB1, MSH2, TGFBR2, NBN, FIGF, BUB1B, CCNB2, RAD51, TOPBP1, CCND2, MAD2L1, PTPRZ1, BLM, FANCD2, RACGAP1, CCNE2, TOP2A, RPLP0, PRC1, NEK2, RBBP4, NEDD9, KRT17, RAD54B, AKAP9, KPNA2, RBBP7, ANAPC7, CENPK, TUBB2B	6.65E-12
Cell cycle	CDK1, PLK1, CHEK1, PTTG1, E2F1, MKI67, BRCA1, CCNA2, BIRC5, CCNB1, TGFBR2, BUB1B, CCNB2, AURKA, CCND2, MAD2L1, BLM, CCNE2, TOP2A, NEK2, CLSPN, AKAP9, LAMA3, ANAPC7, CENPK	5.96E-11
Double Strand DNA	BRCA1, MSH2, NBN, RAD51, TOPBP1, BLM,	0.000152
Homologous Repair	FANCD2, TOP2A, RAD54B	
Histone Phosphorylation	ROCK1, CDK1, MSH2, NBN, AURKA, TOPBP1, BLM, FANCD2, TOP2A, RAD54B	0.000305
Single-Strand Base Excision DNA Repair	MSH2, NBN, TOPBP1, BLM, FANCD2, TOP2A, RAD54B	0.000315
Single-Strand Mismatch DNA Repair	MSH2, NBN, TOPBP1, BLM, FANCD2, TOP2A, RAD54B	0.000405
Direct DNA Repair	MSH2, NBN, TOPBP1, BLM, FANCD2, TOP2A, RAD54B	0.000492
Double Strand DNA Non-Homologous Repair	MSH2, NBN, TOPBP1, BLM, FANCD2, TOP2A, RAD54B	0.000677
Hedgehog Pathway	INHBA, MSH2, NBN, TOPBP1, CCND2, BLM, FANCD2, TOP2A, RBBP4, RAD54B, RBBP7, ANAPC7	0.003321
TRRAP/Tip60 Chromatin Remodeling	MSH2, NBN, TOPBP1, MAD2L1, BLM, FANCD2, TOP2A, RBBP4, RAD54B, RBBP7	0.013707
Apoptosis Regulation	INHBA, BIRC5, MSH2, TGFBR2, NBN, TOPBP1, BLM, FANCD2, TOP2A, RAD54B	0.024945

DISCUSSION

Microarray studies have been extensively carried out over the past few years to explore the gene expression landscapes in breast cancer tissues. This technology enables researchers to study thousands of genes simultaneously and compare the differentially expressed genes in an independent manner. Genes identified from this study could serve as important prognostic indicators and be a guide for the treatment of breast cancer. There are limited local data that explores the complexity of this cancer. To understand the role of differentially expressed genes and elucidating the molecular pathways involved in breast cancer carcinogenesis, whole genome gene expression profile was performed in this study.

We identified 404 up-regulated and 463 down-regulated genes. Among the top ten significant up-regulated genes, three genes were associated with breast cancer, including *CENPF*, *DTL* and *MK167* (22-24). *CENPF* gene is associated with cell proliferation (25). It encodes for kinetochore-associated protein that is involved in the regulation of cell division (26). Overexpression of *CENPF*

may lead to deregulation of cell division and uncontrolled proliferation. A similar result was reported in a previous study and the upregulation of *CENPF* was found to be associated with poor prognosis, poor survival and a decrease in metastasis-free survival (22). On the other hand, *DTL* was found to be highly expressed not only in breast cancer but also in other cancers (23). It might be a potential molecular target for breast cancer treatment as silencing of this gene may cause failure in cytokinesis and induced cell death (23). *MK167*, which is an antigen identified by the monoclonal antibody Ki-67 is a common proliferative marker in breast cancer (24). Its expression has been reported to be high in breast cancer and could result in an uncoordinated cell growth and tumorigenicity (27).

For the top ten significantly down-regulated genes, *HOXA5* and *NRG1* were found to be associated with breast cancer (28, 29). *HOXA5* is a transcription factor that is involved in apoptosis induction (30). Down-regulation of this gene may lead to cell cycle aberration and this could promote breast cancer carcinogenesis (28). Meanwhile, *NRG1* is a tumour suppressor gene and its expression can stimulate apoptosis (29, 31). *NRG1* gene was found to be

frequently silenced in many breast cancers (29) including the samples in our current study.

We further compared our results with the genes listed in the Mammaprint assay (Agendia, Amsterdam, The Netherlands) to check whether there were any overlapping genes. A total eight genes (8 out of 74 genes in the assay) including *C9orf30, CCNE2, CENPA, DTL, ECT2, MELK, NUSAP1, PRC1* and *DIAPH3* were identified to be overlapped. Notably, all of these genes were overexpressed in our samples and their biological functions reflect the hallmarks of cancer as shown in a previous report (32). There are eight hallmarks of cancer including evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, tissue invasion and metastasis, sustained angiogenesis, deregulating cellular energetic and avoiding immune destruction (33, 34). Interestingly, most of the overlapped genes (*CCNE2, CENPA, DTL, ECT2, NUSAP1* and *PRC1*) were involved in the hallmark of limitless replicative potential. Aberration in the expression of these genes would contribute to the uncontrolled cell cycle (32). Meanwhile, *MELK* reflects the hallmark of evading apoptosis, insensitivity to antigrowth signal and self-sufficiency in growth signal (32). Overexpression of this gene could lead to abnormal proliferation and oncogenic transformation during breast carcinogenesis (32). *DIAPH3* was included under the hallmark of tissue invasion and metastasis (32). It regulates the dynamics of microtubules, actin remodelling and cell movement that lead to the escape of cells to distant sites (32, 35).

We also compared our results with the genes listed in the Oncotype DX assay (Genomic Health, Redwood City, California). Out of 21 genes in the assay, three genes (*CCNB1, MYBL2* and *MK167*) were found to overlap. *CCNB1* is involved in mitosis regulation (36) and high expression leads to abnormal mitosis which may contribute to breast cancer progression. Overexpression of this gene was also associated with poor survival in breast cancer (37). Meanwhile, *MYBL2* is a transcription factor that regulates the expression of genes involved in cancer progression (38).

In general, aberration in the molecular pathways such as cell cycle regulation could lead to the uncontrolled cell proliferation. In the current study, most of the clustered genes were involved in the cell cycle regulation. Genes such as *CCNB1, E2F1* and *CDK1* are known to be crucial genes that regulate cell cycle and promote breast carcinogenesis (39, 40). *BLM* is involved in all the pathways shown in Table 2. A previous study showed that *BLM* plays an important role in the homologous recombination pathway for DNA double-strand break repair (41). In addition, genes such *TOP2A* and *TOPBP1* were involved in several pathways that are associated with DNA repair. These genes were reported to be associated with breast cancer (42, 43). Aberration in these genes might cause failure in the DNA repair, aberrant DNA replication and disturbance in cell division in breast cancer.

In conclusion, our study successfully examined the whole genome gene expression profile in breast cancer from our local Malaysian patients. These findings highlighted the significant genes and revealed the biological pathways that could contribute additional knowledge to understand the underlying breast carcinogenesis.

ACKNOWLEDGEMENT

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