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1. Introduction

Breast cancer is the most common cancer in women in the world [1]. According to the most recent estimates from GLOBOCAN published by the International Agency for Research on Cancer (IARC) [2], there were nearly 1.7 million new breast cancer cases diagnosed in 2012 (25.2% of all cancers in women) and 6.3 millions have been diagnosed with breast cancer in 2007-2012. Breast cancer incidence has been increasing by more than 20% and mortality increased by 14% since 2008 and is the most common cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total). Breast cancer is less favorable in the underdeveloped countries due to less advanced medical diagnosis and treatments. Therefore a good diagnosis/prognosis would help to prevent as well as provide effective clinical treatments.

Biomarker testing is an essential step in the evaluation of breast cancer and help medical doctors and patients in deciding the best treatment strategy. There are several commercial products or services developed towards this purpose. The Oncotype DX (Genomic Health) measures the expression levels of 21 genes and is most helpful for patients of early stage breast cancer with estrogen receptor (ER) positivity and no cancer cells in the lymph node. The HERmark assay (Monogram Biosciences) can quantitatively measure the HER2 total proteins with greater sensitivity than immunohistochemistry (IHC) which is an important indicator of predicting response of HER2-positive breast cancer patients to trastuzumab therapy. There are also tests for BRCA1 and BRCA2 mutations for the hereditary breast cancer patients. The targeted sequencing-based breast cancer panels such as BreastNext (Ambry Genetics) and BROCA (University of Washington) can be used to screen for mutations and copy number variants in genes implicated in breast cancer, including BRCA1 and BRCA2.
Despite the relative success of these tests, there is a need for more efficient biomarkers in specific groups of breast cancer, such as lobular carcinoma [3, 4], triple-negative breast cancer [5] and early-onset breast cancers [6, 7] for diagnostic and/or prognostic application. We believe the discovery of more useful markers using the wealth of gene expression data available publicly nowadays would help medical doctors in the decision of the best way to help breast cancer patients, especially if the markers are correlated with specific therapeutic interventions. In the past decade, the high throughput microarray technique has been widely used to identify potential biomarkers for various cancers [8-12]. Recent years, the employment of RNA sequencing (RNA-seq) allows researchers to obtain transcriptome information and differential gene expression profiling at a much higher resolution. With the huge amount of data generated by these technologies, we are able to study the association of genes with cancer survival and identify novel potentially prognostic biomarkers for cancers with improved estimation. Traditionally, genetic search identifies genes that correlate with poor or good prognosis of patients. However, it is important to consider the epistatic gene-gene interactions underlying gene expression in complex diseases such as cancer. The epistatic (second or higher order) information would allow more refined prognostic evaluation that may help clinical treatments. Furthermore, epistatic analysis could be useful for identifying hub genes involved in prognosis and help to identify the major genetic risk factors and pathways in breast cancer.

In this work, we utilized the breast tumor RNA-seq data from The Cancer Genome Atlas (TCGA) as well as microarray-based expression datasets from Gene Expression Omnibus (GEO) to detect differentially expressed genes in various subsets of breast cancer patients, to identify genes whose expression profile is associated with survival of breast cancer patients and to examine the influence of co-expression of a second gene in the survival of patients. This analysis identifies specific gene groups differentially expressed between early-onset vs. late-onset breast cancer, between ductal vs. lobular carcinoma, between early vs. advanced stage breast tumors and tumor of various receptor status. Furthermore, epistatic interactions among these genes demonstrate the gene-gene interactions in patient survival and identify several hub genes that may be important determinants of breast cancer.

2. Statistical analysis of gene expression data

A global change in gene expression is a common theme in many human cancers. High-throughput techniques such as microarrays and next generation sequencing allow investigators to observe and compare the transcriptional landscapes of tumor cells in different biological states [13-18]. In this work, we integrated multiple gene expression data from several large-scale breast cancer studies to improve the assessment of differential gene expression in breast tumor cells and to effectively increase statistical power.

We collected 3,188 breast cancer related Affymetrix expression microarray data from GEO (http://www.ncbi.nlm.nih.gov/geo) from the following 16 series: GSE2603, GSE4922, GSE2990, GSE3494, GSE6532, GSE9195, GSE7390, GSE20194, GSE20271, GSE20685, GSE25066, GSE16391, GSE19615, GSE42568, GSE45255 and GSE50948. We also obtained 1,172 breast
invasive carcinoma (BRCA) RNA-seq Level 3 data from TCGA Data Portal (http://tcga-data.nci.nih.gov/). The demographic and clinicopathological characteristics of the breast cancer patients from each study were also retrieved.

2.1. Processing of gene expression data and differential gene expression analysis

The CEL files obtained from microarray experiments were pre-processed by subjecting to quality check using Bioconductor in the R environment to ensure comparability between the different series and microarray platforms. The following quality measurements from the `simpleaffy` and `affy` packages were performed: average background (avbg), scale factor (sfs), percent present (percent.present), and possible RNA Degradation (AffyRNAdeg) of the array. Additionally, the relative log expression (RLE) and normalized unscaled standard error (NUSE) was also estimated using the `affyPLM` package. 466 arrays that did not pass the quality control tests were removed. For the 2,722 arrays that had sufficient quality, the quantile normalization and background correction were performed using the justRMA (robust multi-array average) algorithm of the `affy` package and the gene (probe set)-level log2-transformed expression values were summarized with Custom CDF file annotations (version 18.0.0. ENSG) [19]. Lastly, the COMBAT method available in the `inSilicoMerging` package was used to remove batch effect when combining the final expression data from the HG-U133A and HG-U133 Plus 2.0 arrays [20]. The RMA-normalized expression values from microarrays and the raw count data from RNA-seq datasets were then analyzed using the `edgeR` package [21]. The differentially expressed genes were selected with a threshold of FDR adjusted \( P \)-value < 0.05.

2.2. Clinicopathological characteristics of breast cancer patients

We include 2,722 breast cancer patients from various microarray-based studies (referred as GEO cohort) and 1,052 breast cancer patients from the TCGA project (referred as TCGA cohort) following differential gene expression analyses (Table 1). All patients were women in the GEO cohort with a median age of 53 years. The patients from the TCGA cohort were older with a median age of 58 years and approximately 96% of patients were women. There was a significant amount of clinicopathological data not available from the GEO cohort as noted in Table 1. In both cohorts, there were more stage I/II breast cancer cases than advanced stage cases, and invasive ductal carcinoma (IDC) being the major histological subtype diagnosed. The data also contained status of tumor receptors such as the estrogen receptor (ER), progesterone receptor (PR) and HER2 which are frequently used prognostic factors to aid therapeutic decisions. Many patients were positive for the ER and/or PR, and/or negative for the HER2 receptor.

| Characteristic | Microarray (n = 2722), % | RNA-seq (n = 1052), % |
|----------------|--------------------------|------------------------|
| Sex            |                          |                        |
| Male           | 0                        | 11                     | 1.0%
| Characteristic               | No. of Patients |        |        |
|-----------------------------|----------------|--------|--------|
|                             | Microarray (n = 2722), % | RNA-seq (n = 1052), % |
| Female                      | 2722           | 85.4%  | 1005   | 95.5% |
| Missing data                | 0              | 0.0%   | 36     | 3.4%  |
| Median Age (range)          |                |        |        |
| Younger than 40             | 300            | 11.0%  | 71     | 6.7%  |
| 40 to 55                    | 1184           | 43.5%  | 365    | 34.7% |
| Older than 55               | 1224           | 45.0%  | 580    | 55.1% |
| Missing data                | 14             | 0.5%   | 36     | 3.4%  |
| Stage                       |                |        |        |
| Early (Stage I and II)      | 1236           | 45.4%  | 751    | 71.4% |
| Late (Stage III and IV)     | 370            | 13.6%  | 246    | 23.4% |
| Missing data                | 1116           | 41.0%  | 55     | 5.2%  |
| Histologic Subtype          |                |        |        |
| IDC                         | 500            | 18.4%  | 754    | 71.7% |
| ILC                         | 32             | 1.2%   | 168    | 16.0% |
| Mixed                       | 47             | 1.7%   | 29     | 2.8%  |
| Others                      | 6              | 0.2%   | 64     | 6.1%  |
| Missing data                | 2137           | 78.5%  | 37     | 3.5%  |
| ER Status                   |                |        |        |
| ER positive                 | 1710           | 62.8%  | 749    | 71.2% |
| ER negative                 | 647            | 23.8%  | 222    | 21.1% |
| Missing data                | 365            | 13.4%  | 81     | 7.7%  |
| PR Status                   |                |        |        |
| PR positive                 | 1017           | 37.4%  | 650    | 61.8% |
| PR negative                 | 684            | 25.1%  | 318    | 30.2% |
| Missing data                | 1021           | 37.5%  | 84     | 8.0%  |
| HER2 Status                 |                |        |        |
| HER2 positive               | 202            | 7.4%   | 150    | 14.3% |
| HER2 negative               | 946            | 34.8%  | 524    | 49.8% |
| Missing data                | 1574           | 57.8%  | 378    | 35.9% |
| Female patients with a least one type of survival data | 2294 | 84.3% | 999 | 36.7% |

Table 1. Patient characteristics of the GEO and TCGA cohorts.
2.3. Differentially expressed genes in patients from different age groups

Differential gene expression analysis was performed to identify over- and under-expressed genes specific to tumors derived from young, middle-aged and elderly breast cancer patients. As presented in Figure 1, there were very few middle-aged-specific expression signatures, indicating that the gene expression pattern of middle-aged patients was not significantly different from the young adults and/or elderly patients. In contrast, the elderly breast cancer patients possessed a high number of differentially expressed genes specific to this age group. IPA analysis of the differentially expressed genes from tumor cells obtained from older patients have decreased cell proliferation, movement, migration and cell cycle progression (activation z-score between -2.677 and -1.611) and increased cell death (activation z-score = 1.321). On the contrary, tumor cells from young patients were predicted to have increased proliferation of cells and DNA synthesis (activation z-score between 2.000 and 2.117) and decreased cell death and apoptosis (activation z-score between -0.586 and -0.299).

It is interesting to note that 14 genes that were over-expressed in young patients were under-expressed in elderly patients, and conversely, 15 genes under-expressed in young patients were over-expressed in elderly patients (Table 2). Several of these genes such as \textit{BIRC5} (survivin), \textit{KPNA2}, \textit{PLAC8} (onzin), \textit{TFF12}, \textit{CITED2}, \textit{NKX3-1}, \textit{PIP} and \textit{ZNF395} have been found to play a role in cancer cell proliferation and cancer progression [23-28].

| Type    | Symbol | Entrez Gene Name   | Location  | Type(s) |
|---------|--------|--------------------|-----------|---------|
| Young-Up| \textit{BIRC5} | baculoviral IAP repeat containing 5 | Cytoplasm | Other   |
|         | \textit{DCX}  | doublecortin        | Cytoplasm | Other   |
| Elderly-Dn| \textit{GAL} | galanin/GMAP prepropeptide | Extracellular Space | Other   |

Figure 1. Number of significantly over- and under-expressed genes in the three age groups presented with the jvenn Venn diagram viewer [22].
| Type | Symbol | Entrez Gene Name | Location | Type(s) |
|------|--------|-----------------|----------|---------|
| Young-Dn | HN1 | hematological and neurological expressed 1 | Nucleus | Other |
| | KPNB2 | karyopherin alpha 2 | Nucleus | Transporter |
| | NOL11 | nucleolar protein 11 | Nucleus | Other |
| | NUP85 | nucleoporin 85kDa | Cytoplasm | Other |
| | PLAC8 | placenta-specific 8 | Nucleus | Other |
| | POLR3G | polymerase (RNA) III (DNA directed) | Nucleus | Enzyme |
| | PSMA4 | proteasome alpha 4 subunit isoform 1 | Cytoplasm | Peptidase |
| | RAPGEFL1 | Rap guanine nucleotide exchange factor | Other | Other |
| | TFPI2 | tissue factor pathway inhibitor 2 | Extracellular Space | Other |
| | UCHL1 | ubiquitin carboxyl-terminal esterase L1 | Cytoplasm | Peptidase |
| | XDH | xanthine dehydrogenase | Cytoplasm | Enzyme |
| Elderly-Up | ABCC6 | ATP-binding cassette, sub-family C, member 6 | Plasma Membrane | Transporter |
| | ACAA1 | acetyl-CoA acyltransferase 1 | Cytoplasm | Enzyme |
| | CCDC28A | coiled-coil domain containing 28A | Other | Other |
| | CITED2 | Cbp/p300-interacting transactivator | Nucleus | Transcription regulator |
| | CLMN | calmin (calponin-like, transmembrane) | Cytoplasm | Other |
| | CTDSP1 | small CTD phosphatase 3 isoform 1 | Nucleus | Other |
| | CTSF | cathepsin F | Cytoplasm | Peptidase |
| | FMO5 | flavin containing monoxygenase 5 | Cytoplasm | Enzyme |
| | GPC4 | glypican 4 | Plasma Membrane | Transmembrane receptor |
| | KIF13B | kinesin family member 13B | Cytoplasm | Other |
| | NDST1 | N-deacetylase/N-sulfotransferase (heparan) | Cytoplasm | Enzyme |
| | NKX3-1 | NK3 homeobox 1 | Nucleus | Transcription regulator |
| | PIP | prolactin-induced protein | Extracellular Space | Peptidase |
| | ZNF385D | zinc finger protein 385D | Nucleus | Other |
| | ZNF395 | zinc finger protein 395 | Cytoplasm | Other |

Table 2. Concordant differentially expressed genes identified in the young and elderly breast cancer patients.

2.4. Differentially expressed genes in patients with early stage versus advanced stage breast cancer

We compared the gene expression profile of patients diagnosed with early stage (stage I and II) breast cancer with those with advanced stage (stage III and IV) breast cancer. We identified 79 over-expressed and 140 under-expressed genes in early stage breast cancer. IPA analysis
showed 121 of the total 219 differentially expressed genes were associated with cancer ($P$-value = 4.81E-02), and 24 were specifically associated with breast cancer ($P$-value = 3.25E-03, Table 3). Also, there were 17 under-expressed genes in early stage breast cancer (i.e. over-expressed in advanced stage tumors) that were found to be cancer recurrence-associated (ADOR3, FLT4, GSR, HSP90AA1, TEK and TXNRD1) and metastasis-associated (ACP5, FLT4, FTL, GSR, HSP90AA1, MAPK11, MMP9, NRAS, PGF, SCD and TEK). Interestingly, we detected over-expression of the DNA methyltransferase DNMT1 in early stage tumors. In cancer cells, the over-expression of this gene can lead to hypermethylation of CpG islands and epigenetically silences multiple tumor suppressor genes and hence promotes tumorigenesis in early stage cancers [29-31].

| Symbol     | Entrez Gene Name                      | Location       | Type(s)          | DE Status |
|------------|---------------------------------------|----------------|-----------------|-----------|
| ACP5       | acid phosphatase 5, tartrate resistant | Cytoplasm      | phosphatase     | Down      |
| APOE       | apolipoprotein E                       | Extracellular Space | transporter    | Down      |
| ARRB1      | arrestin, beta 1                       | Cytoplasm      | other           | Down      |
| CDKN1A     | cyclin-dependent kinase inhibitor 1A   | Nucleus        | other           | Down      |
| ETV1       | ets variant 1                          | Nucleus        | transcription regulator | Down      |
| FLT4       | fms-related tyrosine kinase 4          | Plasma Membrane | transmembrane receptor | Down      |
| GPC3       | glypican 3                             | Plasma Membrane | other           | Up        |
| GPR126     | G protein-coupled receptor 126         | Plasma Membrane | G-protein coupled receptor | Down      |
| HBB        | hemoglobin, beta                       | Cytoplasm      | transporter     | Down      |
| HIC1       | hypermethylated in cancer 1            | Nucleus        | transcription regulator | Down      |
| HSP90AA1   | heat shock protein 90kDa alpha         | Cytoplasm      | enzyme          | Down      |
| HSPB7      | cardiovascular heat shock protein      | Cytoplasm      | other           | Down      |
| MMP15      | matrix metalloproteinase 15 preproprotein | Extracellular Space | peptidase    | Down      |
| MMP28      | matrix metalloproteinase 28 isoform 1  | Extracellular Space | peptidase    | Down      |
| MMP9       | matrix metalloproteinase 9 preproprotein | Extracellular Space | peptidase    | Down      |
| NOS3       | nitric oxide synthase 3 (endothelial cell) | Cytoplasm      | enzyme          | Down      |
| PPM1D      | protein phosphatase 1D                 | Cytoplasm      | phosphatase     | Down      |
| PSIP1      | PC4 and SFRS1 interacting protein 1    | Nucleus        | other           | Up        |
| PXN        | paxillin                               | Cytoplasm      | other           | Down      |
| S100A2     | S100 calcium binding protein A2        | Nucleus        | other           | Down      |
| SEL1       | selectin L                             | Plasma Membrane | transmembrane receptor | Down      |
| TAL1       | T-cell acute lymphocytic leukemia 1    | Nucleus        | transcription regulator | Down      |
| TEK        | TEK tyrosine kinase, endothelial       | Plasma Membrane | kinase         | Down      |
| TNC        | tenascin C                             | Extracellular Space | other         | Up        |

Table 3. The 25 differentially expressed genes associated with breast cancer in the early versus advanced stage analysis.
2.5. Differentially expressed genes in patients with Invasive Ductal Carcinoma (IDC) versus Invasive Lobular Carcinoma (ILC)

The invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) are the two major histological subtypes of breast cancer. They also represented the main types of breast cancer cases gathered in this study. We compared the gene expression profiles of patients with IDC and ILC and identified 216 over-expressed and 126 under-expressed genes in IDC as compared to patients with ILC. IPA analysis showed 66 genes were related to breast cancer (Table 4), including 12 transcription regulators (ATF3, BTG2, EGR1, EZH2, FOS, FOSB, JUN, JUNB, MTDH, STAT1, ZFP36 and ZNF423) and a translation regulator (EIF4EBP1). There were also 12 genes annotated as tumor suppressor genes in the TSGene database [32], where CDH1, DKK1 and S100A2 were over-expressed in IDC and BTG2, CAV1, EGR1, GPC3, MUC1, NR4A1, SLIT2, TGFB2 and ZFP36 were under-expressed in IDC. IPA predicted common upstream regulators KDM5B, STUB1, CDKN1A, HIF1A and TGFB1 to be inhibited whereas FOXM1, IFNB1, IFNG and PPARG were in activated states. Additionally, the activities of several disease functions such as cell proliferation, invasion and DNA replication were predicted to be increased in IDC (activation z-score between 1.342 and 3.092).

| Symbol       | Entrez Gene Name | Location                     | Type(s)                | DE Status |
|--------------|------------------|------------------------------|------------------------|-----------|
| ACACB        | acetyl-CoA carboxylase beta | Cytoplasm enzyme | Down                  |
| ALDH1A1      | aldehyde dehydrogenase 1 family, member A1 | Cytoplasm enzyme | Down                  |
| APOBEC3B     | apolipoprotein B mRNA editing enzyme, catalytic | Cytoplasm enzyme | Up                     |
| ATF3         | activating transcription factor 3 | Nucleus transcription regulator | Down                  |
| BIRC5        | baculoviral IAP repeat containing 5 | Cytoplasm other | Up                     |
| BTG2         | BTG family, member 2 | Nucleus transcription regulator | Down                  |
| CAV1         | caveolin 1, caveolin protein, 22kDa | Plasma Membrane transmembrane receptor | Down                  |
| CCL21        | chemokine (C-C motif) ligand 21 | Extracellular Space cytokine | Down                  |
| CD34         | CD34 molecule | Plasma Membrane | other                  |
| CD69         | CD69 molecule | Plasma Membrane transmembrane receptor | Down                  |
| CDC20        | cell division cycle 20 | Nucleus other | Up                     |
| CDH1         | cadherin 1, type 1, E-cadherin (epithelial) | Plasma Membrane | other                  |
| CDH3         | cadherin 3, type 1, P-cadherin (placental) | Plasma Membrane | other                  |
| CDH5         | cadherin 5, type 2 (vascular endothelium) | Plasma Membrane | other                  |
| CDK1         | cyclin-dependent kinase 1 | Nucleus kinase | Up                     |
| Symbol | Entrez Gene Name | Location | Type(s) | DE Status |
|--------|-----------------|----------|---------|-----------|
| CXCL14 | chemokine (C-X-C motif) ligand 14 | Extracellular Space | cytokine | Down |
| CXCL2  | chemokine (C-X-C motif) ligand 2 | Extracellular Space | cytokine | Down |
| CYR61  | cysteine-rich, angiogenic inducer, 61 | Extracellular Space | other | Down |
| DKK1   | dickkopf WNT signaling pathway inhibitor 1 | Extracellular Space | growth factor | Up |
| DSCC1  | DNA replication and sister chromatid cohesion 1 | Nucleus | other | Up |
| DUSP1  | dual specificity phosphatase 1 | Nucleus | phosphatase | Down |
| EGR1   | early growth response 1 | Nucleus | transcription regulator | Down |
| EIF4EBP1 | eukaryotic translation initiation factor 4E | Cytoplasm | translation regulator | Up |
| EZH2   | enhancer of zeste homolog 2 (Drosophila) | Nucleus | transcription regulator | Up |
| FABP7  | fatty acid binding protein 7, brain | Cytoplasm | transporter | Up |
| FOS    | FBJ murine osteosarcoma viral oncogene homolog | Nucleus | transcription regulator | Down |
| FOSB   | FBJ murine osteosarcoma viral oncogene homolog B | Nucleus | transcription regulator | Down |
| GPC3   | glypican 3 | Plasma Membrane | other | Down |
| GRB7   | growth factor receptor-bound protein 7 | Plasma Membrane | other | Up |
| HSPB8  | heat shock 22kDa protein 8 | Cytoplasm | kinase | Up |
| IER2   | immediate early response 2 | Cytoplasm | other | Down |
| IGF1   | insulin-like growth factor 1 (somatomedin C) | Extracellular Space | growth factor | Down |
| IGFBP6 | insulin-like growth factor binding protein 6 | Extracellular Space | other | Down |
| ITIH5  | inter-alpha trypsin inhibitor heavy chain | Other | other | Down |
| JUN    | jun proto-oncogene | Nucleus | transcription regulator | Down |
| JUNB   | jun B proto-oncogene | Nucleus | transcription regulator | Down |
| KPNA2  | karyopherin alpha 2 | Nucleus | transporter | Up |
| KRT6B  | keratin 6B | Cytoplasm | other | Up |
| MMP1   | matrix metalloproteinase 1 preproprotein | Extracellular Space | peptidase | Up |
| MMP9   | matrix metalloproteinase 9 preproprotein | Extracellular Space | peptidase | Up |
| MRPL13 | mitochondrial ribosomal protein L13 | Cytoplasm | other | Up |
| MRPL15 | mitochondrial ribosomal protein L15 | Cytoplasm | other | Up |
| MTDH   | metadherin | Cytoplasm | transcription regulator | Up |
| Symbol | Entrez Gene Name | Location | Type(s) | DE Status |
|--------|-----------------|----------|---------|-----------|
| MUC1   | mucin 1, cell surface associated | Plasma Membrane | other | Down |
| NR4A1  | nuclear receptor subfamily 4, group A, member 1 | Nucleus | ligand-dependent nuclear receptor | Down |
| ORM1   | orosomucoid 1 | Extracellular Space | other | Up |
| PCK1   | pyruvate dehydrogenase kinase, isozyme 4 | Cytoplasm | enzyme | Up |
| RFC1   | replication factor C (activator 1) 4, 37kDa | Nucleus | other | Up |
| RRM2   | ribonucleotide reductase M2 | Nucleus | enzyme | Up |
| S100A2 | S100 calcium binding protein A2 | Nucleus | other | Up |
| S1IT2  | slit homolog 2 (Drosophila) | Extracellular Space | other | Down |
| SPPI   | secreted phosphoprotein 1 | Extracellular Space | cytokine | Up |
| SQLE   | squalene epoxidase | Cytoplasm | enzyme | Up |
| STAT1  | signal transducer and activator of transcription | Nucleus | transcription regulator | Up |
| TCP1   | t-complex 1 | Cytoplasm | other | Up |
| TGFBR2 | transforming growth factor, beta receptor II | Plasma Membrane | kinase | Down |
| TIMP4  | TIMP metalloproteinase inhibitor 4 | Extracellular Space | other | Down |
| TOP2A  | topoisomerase (DNA) II alpha 170kDa | Nucleus | enzyme | Up |
| TP53   | tumor protein D52 | Cytoplasm | other | Up |
| TYMS   | thymidylate synthetase | Nucleus | enzyme | Up |
| UBE2C  | ubiquitin-conjugating enzyme E2C | Cytoplasm | enzyme | Up |
| VEGFA  | vascular endothelial growth factor A | Extracellular Space | growth factor | Up |
| ZFP36  | ZFP36 ring finger protein | Nucleus | transcription regulator | Down |
| ZNF423 | zinc finger protein 423 | Nucleus | transcription regulator | Down |

Table 4. The 66 differentially expressed genes associated with breast cancer in the IDC versus ILC analysis.

2.6. Differentially expressed genes in patients with different receptor status

In the last part of the differential gene expression analysis, we sought to examine the differentially expressed genes of breast cancer patients of different receptor status: (1) estrogen receptor positive (ER+) versus ER negative (ER−), (2) progesterone receptor positive (PR+) versus PR negative (PR−), (3) HER2 receptor positive (HER2+) versus HER2 negative (HER2−), and (4) triple-negative breast cancer (TNBC, also known as basal-like breast cancer) versus
non-TNBC. The Venn diagram in Figure 2 summarized the intersections between the differentially expressed genes identified in the four assays. There were 57% and 65% of breast cancer patients that were both ER+ and PR+ in the GEO and TCGA cohorts respectively; hence the patient pools divided by the ER positivity for gene expression assays are similar to that divided by the PR positivity. Because of this fact, it is not surprising to observe genes that were found over- or under-expressed in the ER assay were also differentially expressed in the same direction in the PR assay. Likewise, genes that were over-expressed in TNBC were under-expressed in the ER and PR assays (n = 74) and ER, PR and HER2 assays (n = 35), and vice versa for the under-expressed genes in TNBC (n = 87 and 2 respectively).

Figure 2. Number of differentially up- and down-regulated genes in the ER, PR, HER2 or TNBC receptor status assays. The GALNT6 (polypeptide N-acetylgalactosaminyltransferase) and SCGB2A2 (secretoglobin) are the two genes consistently over-expressed in ER+, PR+, HER2+ breast tumors but under-expressed in TNBC. There were also 87 genes over-expressed in ER+ and PR+ breast tumors and under-expressed in TNBC, including seven transcription regulators (EGR3, FOXA1, GATA3, INSM1, NRIP1, TBX3 and XBPI) and 11 breast cancer associated genes (ABAT, AGR2, CXCL14, GSTM3, HSPB8, MUC1, NR4A2, PGR,PIP, PLAT and PSD3). On the other hand, there were more under-expressed genes (n = 35) shared among ER+, PR+ and HER2+ breast tumors that were over-expressed in TNBC. Among these are four transcription regulators (ELF5, EN1, FOXC1 and ZIC1) and 12 extracellular proteins (CHI3L1, CHI3L2, COL2A1, COL9A3, CRLF1, KLK6, KLK7, MMP12, MMP7, PTX3, SERPINB5 and SOSTDC1), and some of these genes are known TNBC-associated markers [33-37].
3. Identifying survival-related genes of patients with breast cancer

In cancer survival analysis, survival time is often defined as the period of time from the beginning of the medical process (treatment, surgery, etc.) until the death (or some other events such as development of a particular symptom or to relapse after the remission of disease) of the observed patient or until the end of observation. The goal of such analysis is to link the time to event (i.e. survival time) to certain explanatory variables. New methodologies were developed for calculating the survival probabilities using gene expression profiles when genome-wide expression data becomes increasingly available in the past two decades [38-42].

In this work, we analyzed associations between breast cancer patient survival and gene expression of breast tumors from published microarray and the RNA-seq datasets, denoted as the GEO and TCGA cohorts respectively. Survival analysis was performed separately for each cohort and the median times from diagnosis to death or last follow-up were 99.5 and 21.4 months in the GEO and TCGA cohorts respectively. We transform the expression values into gene expression status (i.e. 0 for low expression and 1 for high expression) using the modified auto_cutoff function of the R script available from the Kaplan Meier-plotter website (http://kmplot.com/). The survival probability is calculated using the “survival” package and modified kmplot function (http://biostat.mc.vanderbilt.edu/wiki/Main/TatsukiRcode#kmplot) is used to plot Kaplan-Meier curves. The hazard ratio with 95% confidence intervals and log-rank P-value are estimated using the Cox proportional hazards model. All analyses were conducted within the R statistical environment.

3.1. Univariate gene selection and survival analysis

We extract the gene expression profiles of 1,694 genes that were found differentially expressed (consistently in microarray and RNA-seq datasets) in any one type of the assays discussed in section 2.3 to 2.6. We calculated the overall survival (OS), relapse-free survival (RFS) and the distant metastasis-free survival (DMFS) of breast cancer patients with respect to the expression status. DMFS is not calculated for the TCGA cohort due to unavailability of the time to distant metastases information from patients in this cohort. The log-rank P-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) method and were used to select genes expression profiles significantly associated with survival.

We summarized the survival statistics, including the hazard ratios (an estimate of the ratio of the hazard rate in the highly versus the lowly expressed patient group) and the estimated 2- and 10-year survival rates in Table 5. There were about 24% OS-associated, 48% RFS-associated and 51% DMFS-associated genes that have adjusted log-rank P-value < 0.01 in the GEO cohort. There were 23% OS-associated genes but only 1.3% RFS-associated genes in the TCGA cohort, due to much fewer relapse/recurrence information in this cohort (adjusted log-rank P-value < 0.05). The breast cancer patients in the TCGA cohorts have lower overall survival (10-year) than those from the GEO and both cohorts have similar RFS rates. In the DMFS analysis, 51% of the differentially expressed genes were significant predictor of DMFS.
### Statistics

| Statistics                              | GEO    | TCGA   |
|----------------------------------------|--------|--------|
| Adjusted log-rank P-value cutoff       | 0.01   | 0.05   |
| Overall survival (OS)                  |        |        |
| No. of genes associated with OS        | 414 (24.4%) | 386 (22.8%) |
| **Hazard Ratio > 1**                   |        |        |
| No. of genes                          | 192    | 134    |
| 2-year survival (low / high expression)| 0.969, 0.923 | 0.968, 0.947 |
| 10-year survival (low / high expression)| 0.798, 0.658 | 0.562, 0.369 |
| **Hazard Ratio < 1**                   |        |        |
| No. of genes                          | 222    | 252    |
| 2-year survival (low / high expression)| 0.918, 0.968 | 0.945, 0.970 |
| 10-year survival (low / high expression)| 0.654, 0.787 | 0.382, 0.558 |
| Relapse-free survival (RFS)            |        |        |
| No. of genes associated with RFS       | 811 (47.8%) | 22 (1.3%) |
| **Hazard Ratio > 1**                   |        |        |
| No. of genes                          | 344    | 7      |
| 2-year survival (low / high expression)| 0.901, 0.840 | 0.949, 0.829 |
| 10-year survival (low / high expression)| 0.685, 0.586 | 0.754, 0.595 |
| **Hazard Ratio < 1**                   |        |        |
| No. of genes                          | 467    | 15     |
| 2-year survival (low / high expression)| 0.845, 0.900 | 0.826, 0.946 |
| 10-year survival (low / high expression)| 0.588, 0.684 | 0.538, 0.749 |
| Distant metastasis-free survival (DMFS)|        |        |
| No. of genes associated with DMFS      | 856 (50.5%) | NA     |
| **Hazard Ratio > 1**                   |        |        |
| No. of genes                          | 384    | NA     |
| 2-year survival (low / high expression)| 0.923, 0.863 | NA     |
| 10-year survival (low / high expression)| 0.755, 0.663 | NA     |
| **Hazard Ratio < 1**                   |        |        |
| No. of genes                          | 472    | NA     |
| 2-year survival (low / high expression)| 0.858, 0.926 | NA     |
| 10-year survival (low / high expression)| 0.667, 0.754 | NA     |

Table 5. Survival statistics according to gene expression profiles of breast cancer patients.

#### 3.2. Cox regression analysis using the expression profiles of two genes

From the three survival data, i.e. OS, RFS and DMFS, we selected the top 500 most significantly survival associated gene expression profiles consistent in both cohorts to generate 124,750 two-gene combinations and perform Cox regression analysis with two covariates (i.e. using the expression status of each gene as a covariate).

There were 81,902 (65.7%) and 78,136 (62.6%) pairs whose expression signatures of both genes remained predictors of OS in the GEO and TCGA cohorts respectively (P-value of the coeffi-
cient estimates < 0.05). Of these, 31,189 pairs were mutual in the two cohorts and 234 gene-pairs (consisting of 131 genes) had survival probability patterns greatly shifted compared with the previous single-gene model. The strongest predictor pairs were \( \text{COL16A1-ARHGEF3} \), \( \text{IGF1R-LTB} \), \( \text{IGF1R-PTGDS} \), \( \text{NPY1R-ARHGEF3} \) and \( \text{SERPIN1-ACADSB} \), where the lower expression of both genes in each pair was associated with lowest survival probabilities in all five cases. The results were presented as Kaplan Meier plots in Figure 3A to E.

Figure 3. The Kaplan Meier plots of five OS-associated gene-pairs that also gained most changes in survival probabilities compared to the matching univariate approach.
The same analysis was also performed for RFS in the GOE and TCGA cohorts, and 85,244 (68.3%) and 64,049 (51.3%) pairs were significant predictors of RFS respectively ($P$-value of the coefficient estimates < 0.05). We found 22,165 significant pairs common in the two cohorts and 1,130 gene-pairs (consisting of 276 genes) whose survival probability patterns had greatly shifted compared with the single-gene model. The most significant RFS-associated pairs were ADM-MYBPC1, DIRAS3-TANC2, KIFC1-ADORA3, PDSS1-DIRAS3, STMN1-ADORA3 (Figure 4).

Figure 4. The Kaplan Meier plots of five RFS-associated gene-pairs that also gained most changes in survival probabilities compared to the matching univariate approach.
Lastly, we also make use of the DMFS data available from the GEO cohort to demonstrate the improvement of epistatic gene-pair approach in predicting survival probabilities. Of the 124,750 two-gene combinations, 122,751 (98.4%) were significant predictors of DMFS in breast cancer patients. The high percentage of strong two-gene predictors derived from the DMFS analysis was most likely due to the already high numbers of strong single-gene predictors as shown in Table 5. We further distinguished 228 gene-pairs (consisting of 138 genes) whose survival probability patterns had greatly shifted compared with the single-gene model. Six most significantly improved DMFS-associated pairs were \textit{MMP15-SPDEF}, \textit{TRIB3-ETV1}, \textit{TRIB3-PLD1}, \textit{TRIB3-TRIM2}, \textit{TRIM2-KRT14} and \textit{XBPI-TRIB3} (Figure 5).

\[\text{Figure 5.} \text{ The Kaplan Meier plots of six DMFS-associated gene-pairs that also gained most changes in survival probabilities compared to the matching univariate approach.}\]
3.3. Differentially expressed survival-associated hub genes and gene-pair candidates

As mentioned in the section 3.2, we have identified 234, 1,130 and 228 OS-, RFS- and DMFS-associated gene-pairs (consisting of 131, 276 and 138 genes respectively) that showed improved predictive performance. Some of these genes may be paired with many partners while remaining highly significant. In Table 6, we list five genes that have high number of pairing possibilities and also common in OS, RFS and DMFS analysis.

| Genes | No. of Gene-pairs | GEO RFS log-rank P | single covariate | multiple covariates |
|-------|-------------------|--------------------|-----------------|---------------------|
| MEOX1 | 6                 | 0                  | 4.94E-08        | 3.43E-11 (C3orf18) ~ 4.02E-08 |
| PPAP2B| 37                | 0                  | 1.27E-04        | 1.07E-10 (ADM) ~ 7.17E-05 |
| PRPF38B| 8               | 0                  | 3.41E-02        | 1.04E-12 (DRAS3) ~ 1.17E-02 |
| SERPINA1 | 7             | 20                 | 3.21E-05        | 2.30E-12 (CDT1) ~ 2.34E-05 |
| XB1   | 0                 | 11                 | 1.97E-03        | 2.47E-13 (DRAS3) ~ 1.11E-03 |

Table 6. Five hub genes that associated with more than one type of survival data.

| Gene-Pairs | OS DE Status of Gene 1 | RFS DE Status of Gene 2 | DMFS DE Status of Gene 1 | RFS DE Status of Gene 2 |
|------------|------------------------|-------------------------|--------------------------|-------------------------|
| C3orf18-PPAP2B | 0.51 0.51 0.66 0.64 - - | Her2+ Down | Elderly Down |
| IGF1R-KLRB1 | 0.39 0.60 0.72 0.73 - - | ER+ Up / TNBC Down | IDC Down |
| NME5-PPAP2B | 0.51 0.56 0.77 0.67 - - | ER+ Up / PR+ Up / Her2+ Down / TNBC Down | Elderly Down |
| PRPF38B-RAMP3 | 0.56 0.58 0.80 0.62 - - | Early Stage Up (i.e. Advanced Stage Down) | ER+ Up / PR+ Up / Her2+ Down / TNBC Down |
| GATA3-SERPINA1 | 0.62 0.41 - - 0.56 0.56 | ER+ Up / PR+ Up / TNBC Down | Young Down / ER+ Up / PR+ Up / Her2+ Down / TNBC Down |
| PSAT1-SERPINA1 | 1.61 0.44 - - 1.53 0.56 | Elderly Down / ER+ Down / PR+ Down / TNBC Up | Young Down / ER+ Up / PR+ Up / Her2+ Down / TNBC Down |
| MMP15-SLC44A4 | - - 1.29 0.75 1.69 0.60 | Early Stage Down (i.e. Advanced Stage Up) | ER+ / TNBC Down |

Table 7. The gene-pairs that associated with more than one type of survival data.

There were also seven gene-pairs that were significantly associated with more than one type of survival data (Table 7). By incorporating the differential expression information we derived previously, we may observe that the TNBC patients were noticeably having worse survival outcomes than non-TNBC patients as TNBC is known to be an aggressive breast cancer subtype [43, 44]. For example, both GATA3 [45, 46] and SERPINA1 were found significantly under-
expressed in TNBC cases and the low expressions of both genes were correlated with poor OS and DMFS. Additionally, the over-expression of PSAT1 and the under-expression of SERPINAI in TNBC patients also correlated with poor OS and DMFS. Moreover, the over-expression of MMP15 relating to advanced stage breast cancer and the under-expression of SLC44A4 associated with TNBC are predictors of cancer recurrence as well as distant metastases.

4. Conclusion and perspectives

In section 2 of this chapter, we identified 1,694 genes that were differentially expressed in breast cancer patients of three age groups, early versus advanced stage breast cancers, invasive ductal versus invasive lobular breast cancers, and patients of various receptor status. While some of these genes are known to participate in the biological and genetic pathways that lead to breast cancer and many are novel findings. In section 3, we showed that more than 20% the differentially expressed genes were associated with at least one type of survival data. Our data indicated improved predictive performance when using a multivariate approach of combining the expression of two genes in the assessment of survival data. Perceivably, the gene pairs found in the epistatic analysis could provide useful pictures in gene interactions in breast carcinogenesis.

Breast cancer is a heterogeneous and complex disease where researchers and doctors have implemented different classifications (be it molecular, pathological, genetic or prognostic) to aid disease diagnosis and treatment decision. In the future, we hope to use the gene expression profiles of multiple survival-associated biomarkers to sub-classify patients of different types of breast cancer, and ultimately allow medical practitioners to derive better disease assessment and treatment decision.

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