Gene Expression Profiles for Recurrence of Lymph Node-positive Primary Breast Cancer in Women Over 40 Years of Age

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Research article

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Abstract

Background:

Specific gene expression profiles correlate with recurrence of breast cancer in lymph node-negative patients. In contrast, insufficient knowledge is available regarding tumor-specific gene expression in patients with lymph node metastasis before surgery. Furthermore, such patients experience cumulative incidences of relapse greater than 50%.

Methods:

Sections of formalin-fixed paraffin embedded (FFPE) were prepared from breast tumors of 37 patients who were followed for at least 5 years. FFPE samples of patients with recurrent ductal breast cancer (n = 25) and 12 FFPE samples of such patients without recurrence were subjected to microarray analysis to identify gene expression profiles specifically associated with positive lymph nodes confirmed during surgery that were accompanied by lymphocytic invasion. Immunohistochemistry was employed to determine the estrogen receptor (ER) status of cancer tissues. All patients were administered tamoxifen after surgery, and this treatment continued for more than 5 years, or until cancer recurred. This strategy eliminated interactions between different therapeutics as potential confounding factors that influenced patients' outcomes.

Results:

Sixteen genes were expressed at significantly higher levels in patients with ER-positive (+) breast cancer with recurrence compared with those without recurrence. Gene Set Enrichment Analysis of The Kyoto Encyclopedia of Genes and Genomes (KEGG) identified 73 genes encoding olfactory receptors included in the “Olfactory transduction” pathway that were enriched in the ER+ recurrence group (FDR $P < 0.05$). The KEGG “Histidine metabolism” and “Retinol metabolism” pathways were enriched in patients with ER-negative (–) breast cancer with recurrence (FDR $P < 0.05$).

Conclusions:

The present study is the first, to our knowledge, to identify 16 genes encoding proteins with diverse functions as well as 73 genes encoding olfactory receptors. These genes may serve as presurgical biomarkers for the recurrence of ER+ breast cancers with lymph node metastasis before surgery. These findings identify potential therapeutic targets for preventing cancer relapse, particularly after lymph nodes metastasis.

Background

Advances in the treatment of breast cancer have decreased the rate of isolated locoregional recurrence [1]. For example, lumpectomy followed by radiotherapy reduces the recurrence of ipsilateral breast tumors by 52% compared with lumpectomy alone, and lumpectomy followed by radiotherapy and tamoxifen

treatment reduces the incidence of recurrence by 32% compared with lumpectomy followed by radiotherapy [2]. Furthermore, recent studies show that the overall response rates of patients with breast cancer treated with immune checkpoint inhibitors are similar to those of numerous solid malignancies, which may identify effective immunotherapy-based combination strategies [3]. Moreover, young age, node positivity, larger tumors, and hormone receptor-negative tumors are associated with a higher risk of recurrence [4, 5]. Tumor biology influences the time interval to isolated locoregional recurrences. For example, the median times to isolated locoregional recurrences of breast cancer, measured over 20 years [6], are as follows: ER-positive (+), 6.8 years; ER-negative (–), 3.6 years [7].

Reverse-transcriptase-polymerase-chain-reaction assays of 21 prospectively selected genes expressed by ER + patients revealed a correlation between the likelihood of distant recurrence with lymph node-negative breast cancer treated with tamoxifen [8]. Furthermore, the 21-Gene Recurrence Score serves as an independent prognosticator for time to first progression and 2-year overall survival experienced by patients aged 21–70 years with de novo stage IV ER + breast cancer [9] as well as for women < 40 years of age with node-negative and limited node-positive breast cancer [10].

A 76-gene signature comprising 60 genes expressed by ER + patients and 16 genes expressed by ER– patients identifies patients at high risk of distant recurrence [11]. Although the overall prognosis of ER– breast cancer is worse compared with that of the ER + subtype, not all patients with ER– breast cancer experience poor clinical outcomes. These prognostic signatures are very useful for determining the risk of recurrence in the ER + subgroup, although they are less informative for ER– and erb-b2 receptor tyrosine kinase 2 + breast cancers [12]. Other microarray studies show the differences in recurrence frequencies associated with gene expression between ER + and ER– patients with breast cancer. The immune response and tumor invasion are associated with patients with ER–/HER2– and HER2 + breast cancer, respectively [13]. The heterogeneity in the clinical outcomes of ER– breast cancer involves differences in the expression levels of genes that encode the components of the complement and immune response pathways, independent of lymphocytic infiltration [14]. Furthermore, among these pathways, the immune response is the most prognostic, as indicated by the expression levels of genes that encode complement C1q A chain (C1QA); immunoglobulin lambda constant 2 (IGLC2), LY9 (lymphocyte antigen 9); TNF receptor superfamily member 17 (TNFRSF17); secreted phosphoprotein 1 (SPP1); X-C motif chemokine ligand 2 (XCL2); and human leukocyte antigen F (HLA-F) [14].

Although these predictors perform well in identifying relapse of patients without involved lymph nodes, they fail to predict patients with positive lymph nodes. Furthermore, these reports analyzed the populations of Europe or the United States. In contrast, limited data are available for populations of Asia. For example, a study conducted in China found that Ki-67 expression levels are significantly associated with early relapse of breast cancer [15].

Here we compared the gene expression profiles of tumor tissue of patients with ductal breast cancer, with or without recurrence, within 5 years after surgery. Both groups of patients had positive lymph nodes confirmed during surgery, which were accompanied by lymphocytic invasion. Thus, we aimed here to

identify gene expression patterns that will contribute to improving therapy designed to prevent the recurrence of breast cancer in patients with lymphocytic invasion who are administered chemotherapy and tamoxifen.

Methods

Study design and subjects

The present study included 37 patients who underwent breast cancer surgery at Huangpu District Central Hospital of Shanghai, China from 2005 to 2008. These patients were followed for more than 5 years. Sections of formalin-fixed paraffin-embedded (FFPE) tissues were prepared from breast tumors. The FFPE tissue samples included those of 25 of patients with ductal breast cancer with recurrence (ER+/ER−; n = 16, n = 9, respectively), and 12 of patients with ductal breast cancer without recurrence (ER+/ER−; n = 6, n = 6, respectively). ER expression was detected using immunohistochemistry employing a monoclonal antibody against ER. Histologic grade was assessed according to the World Health Organization criteria. All patients had positive lymph nodes confirmed during surgery, which were accompanied by lymphocytic invasion. These patients were administered tamoxifen starting immediately after surgery and continuing for more than 5 years or until cancer recurrence. All patients received chemotherapy, thereby eliminating potential interactions between different treatments to exclude the influence of potential confounding factors on outcomes.

RNA extraction, microarray hybridization, and data analysis

A RecoverAll Total Nucleic Acid Isolation Kit AM1975 (Thermo Scientific, USA) was used to extract total RNA from FFPE tissues according to the manufacturer's protocol. The process mainly involved deparaffinization, protease digestion, nucleic acid isolation, DNase digestion, and final purification. RNA purity and quantification were evaluated using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Gene expression profiles were determined using an Affymetrix Human Clariom D Assay (OE Biotechnology Co., Ltd., Shanghai, China). Sample labeling, microarray hybridization and washing were performed according to the manufacturer's standard protocols. Briefly, total RNAs were reverse-transcribed and purified. Next, the second-cycle cDNAs were fragmented and labeled with biotin, and the labeled cDNA samples were used to probe the microarrays. After washing and staining, the microarrays were scanned using an Affymetrix Scanner 3000 (Affymetrix).

Affymetrix GeneChip Command Console software (version 4.0, Affymetrix) was used to extract raw microarray data, and Expression Console software (version 1.3.1, Affymetrix) was used to perform Robust Multi-array Average (RMA) normalization for gene expression analysis. Differentially expressed genes were identified using GeneSpring software (version 13.1, Agilent Technologies). The fold-change in expression level was used to compare groups of microarray samples. Heat maps generated using the

clustermap function of the Seaborn package in Python visualized gene expression levels among samples. The microarray data were deposited in the Gene Expression Omnibus (GEO) database (accession code GSE183231).

Gene set enrichment analysis (GSEA)

GSEA [16] ranks all genes in a dataset according to their differential expression. To test the significance of a gene set, an enrichment score is defined as the maximum distance from the middle of the ranked list. Thus, the enrichment score indicates whether the genes contained in a gene set are clustered toward the beginning or end of the ranked list. Self-contained and competitive hypothesis tests are conducted using GSEA by altering randomization for hypothesis testing. To develop a self-contained hypothesis, the phenotype labels are permuted; in contrast, the genes are permuted for a competitive hypothesis. We performed 1000 permutations to estimate empirical *P* values of the gene sets. The data were acquired from the GSEA Molecular Signatures Database v7.4 (http://www.gsea-msigdb.org/gsea/msigdb/index.jsp). The threshold for identifying significant pathways and entries was defined by the false discovery rate FDR ($P < 0.05$) and the normalized enrichment score |NES| > 1.

Results

Clinical and pathological features of breast cancer patients

Among 37 patients included in the present study, 11 showed evidence of distant metastasis, 14 showed local recurrence, 12 died after a previous relapse, and 3 patients died without evidence of disease. The 16 ER + patients who relapsed and the 9 ER– patients were followed for 1482 days and 1115 days, respectively. Clinical and pathological features of the patients are summarized in Table 1. The ER + and ER– recurrence groups (local and distant) had slightly higher proportions of larger tumors compared with those of the nonrecurrence ER + and ER– control groups, respectively. There were no significant differences among the groups in age and number of positive lymph nodes observed during surgery.

Table 1
Clinical and pathological features of 37 patients in surgery

	ER + recurrence recurrence (local and distant)	ER + nonrecurrence control	ER-recurrence recurrence (local and distant)	ER- nonrecurrence control
Subjects (n)	16	6	9	6
Age in surgery (year)	56 ± 13	53 ± 6	56 ± 12	56 ± 11
Time to first recurrence (days)	1482 ± 650	-	1115 ± 754	-
lymphocytic invasion +/++ (n)	10 /6	5 /1	3 /6	4 /2
positive nodes	5.8 ± 7.1	5.2 ± 2.7	4.8 ± 9.6	4.0 ± 5.0
Chemotherapy post surgery	√	√	√	√
Dead (n)	8	2	4	2
Premenopausal/ Postmenopausal/ Lost (n)	3/9/4	0/4/2	2/4/3	2/0/4
Tumor size > 20mm (n)	9	1	5	2
tumor size ≦ 20mm (n)	14	5	4	2
Tumor size (mm)	29.3 ± 15.1	24.5 ± 5.9	31.0 ± 15.3	21.2 ± 10.2
Tamoxifen	used	used	used	used
grade 3 / (grade 2and 1)	0.30	0.00	0.55	1.00
ER-positive > 10% positive tumour cells.				

Gene expression profiling of breast cancer tissues

Gene expression profiles were determined using RNA samples from breast tumor tissues of patients as follows: 16 ER + and 6 ER + with or without recurrence, respectively, 9 ER– with recurrence, and 6 ER– without recurrence. The Affymetrix Clariom D Human Assay containing 135,750 probe sets, including 18,858 Entrez Gene RNAs and 66,845 lncRNAs. Principal component analysis (PCoA) revealed that the difference between the expression profiles of ER– with or without recurrence was greater than that between the corresponding two groups of ER + samples (Fig. 1).

Differences in gene expression profiles between ER + tumor tissues of patients with and without recurrence with positive lymph nodes

When we arbitrarily defined a differentially expressed gene (DEG) according to a > 1.5-fold change in expression compared with the reference ($P < 0.05$), we identified 1962 DEGs between the ER + with recurrence group compared with the ER + without recurrence group. Among 771 upregulated DEGs, including 116 coding genes, 392 noncoding genes, and 263 other genes, aspartate beta-hydroxylase (ASPH) was expressed at significantly higher levels (2.75-fold) in the ER + recurrence group compared with the ER + nonrecurrence group ($P < 0.05$). Phospholipase A2, group V (PLA2G2) was expressed at a significantly higher level (2.06-fold in the ER + recurrence group compared with the ER + nonrecurrence group, $P < 0.05$). Among 1191 downregulated DEGs expressed by the ER + recurrence and ER + nonrecurrence groups, we identified the DEGs as follows: 203 coding, 555 noncoding, and 433 others (Fig. 2A, B). Part of the upregulated coding DEGs are shown in Table 2.

Table 2
Sixteen significantly different-expressed coding genes between ER positive samples with recurrence vs ER positive samples without recurrence (up-regulated fold change > 1.5, p value < 0.05)

Fold Change	P-val	FDR P-val	Gene Symbol	Description
2.75	0.0005	0.4937	ASPH	Aspartate beta-hydroxylase
2.06	0.0000	0.2176	PDCD6IP	programmed cell death 6 interacting protein
2.06	0.0049	0.6367	PLA2G5	phospholipase A2, group V
2.04	0.0028	0.6035	PDIA6	protein disulfide isomerase family A, member 6
2.03	0.0409	0.7896	ATP5I	ATP synthase, H + transporting, mitochondrial Fo complex subunit E
1.95	0.0001	0.3837	DCP1B	decapping mRNA 1B
1.92	0.0138	0.7081	MTF2	metal response element binding transcription factor 2
1.70	0.0028	0.6035	GLS	glutaminase
1.69	0.0031	0.6135	OR7E101P	olfactory receptor, family 7, subfamily E, member 101 pseudogene
1.61	0.0039	0.6215	ALG10	ALG10, alpha-1,2-glucosyltransferase
1.60	0.0038	0.6182	KLKB1	kallikrein B1
1.59	0.0027	0.602	TEKT3	tektin 3
1.57	0.0031	0.6135	DHX8	DEAH (Asp-Glu-Ala-His) box polypeptide 8
1.53	0.0150	0.716	PIEZO2	piezo-type mechanosensitive ion channel component 2
1.51	0.0039	0.6215	CNTN1	contactin 1
1.50	0.0109	0.6962	MCUR1	mitochondrial calcium uniporter regulator 1

GO enrichment analysis revealed that the DEGs were associated with the GO terms as follows: “Biological Process” (n = 57), “Cellular Component” (n = 17), and “Molecular Function” (n = 22). The top 20 GO terms are shown in Fig. 3A. The enriched pathways included 14 KEGG pathways associated with all DEGs (Fig. 3B). GSEA identified enriched KEGG pathways significantly associated with the recurrence group as follows: "Olfactory transduction" (FDR, $P = 0.035$; NES = 1.45), as well as the enriched GO term "DNA binding transcription factor activity" (FDR, $P = 0.014$, NES = − 2.03) that was associated with the nonrecurrence group (Fig. 3C, D).

Differences in gene expression profiles between lymph node-positive ER– tumor tissue of patients with or without recurrence

We identified 3833 DEGs between the ER– groups with or without recurrence, among which 1761 were upregulated and 2072 were downregulated (Fig. 4A). The DEGs included 208 coding genes, 934 noncoding genes, and 619 other genes. The latter DEGs included 343 coding genes, 815 noncoding genes, and 914 other genes (Fig. 4B).

GO enrichment analysis revealed that the DEGs were associated with 89 GO terms, including 51 in “Biological Process”, 20 in “Cellular Component.” and 18 in “Molecular Function”. Furthermore, there were 36 KEGG pathways associated with the DEGs between the ER– recurrence and nonrecurrence groups (Supplemental file S1).

GSEA identified the significantly enriched KEGG pathways "Histidine metabolism" and "Retinol metabolism" associated with the recurrence group (FDR $P < 0.05$, NES > 1.0) (Fig. 5A, B), and eight enriched GO terms were significantly associated with the nonrecurrence group (FDR $P < 0.05$, NES < − 2.0) (Supplemental file 2).

Discussion

Few reports contribute insights regarding predictors of outcomes or treatment targets applicable to women aged over 40 years with lymph node-positive primary breast cancer studied here. The 55% probability of recurrence associated with positive lymph nodes after surgery emphasizes the importance of developing more effective biomarkers [17]. Here we show, for the first time to our knowledge, that 16 genes encoding proteins with diverse functions and 73 genes encoding olfactory receptors may serve as biomarkers of the recurrence of breast cancer with the ER + phenotype accompanied by lymph nodes metastasis before surgery.

Diverse human tissues express olfactory receptors that contribute to physiological processes such as cell migration, proliferation and secretion and serve as biomarkers for carcinomas of the prostate, lung, small intestine and breast [18]. Olfactory receptor family 51 subfamily E member 2 (OR51E2) serves as a biomarker for prostate cancer. OR51E2 is also called prostate-specific G-protein-coupled receptor (PSGR),

because it is upregulated in prostate cancer [19, 20]. OR51E1, which is a paralog of OR51E2, serves as a potential biomarker for small intestine neuroendocrine carcinomas [21] and certain types of lung cancer [22]. Moreover, 111 olfactory receptors are detectable in testis, including specific expression of olfactory receptor family 4 subfamily N member 4 (OR4N4), and several other olfactory receptors are expressed in Epstein–Barr virus-transfected lymphocytes [23]. The present study is the first, to our knowledge, to report a correlation between the expression of olfactory receptor genes (73 gene set) and tumor recurrence of ER + breast patients with breast cancer with lymph nodes metastasis. This discovery suggests that olfactory receptors may serve as predictors of recurrence of ER + breast cancer, particularly subsequent to lymph node metastasis.

ASPH is highly expressed during fetal development and in placental trophoblasts, but not in healthy adult human tissues. Furthermore, ASPH is specifically upregulated in cancer cells and is overexpressed in more than 20 different solid neoplasms, in which it contributes to the malignant phenotype. Moreover, ASPH is associated with increased cell proliferation, invasiveness, and poor prognosis. Metastasis is a major cause of death of patients with cancer, and new antimetastatic strategies are required. Numerous pathways contribute to cell migration and invasion of normal tissues by malignant tumors. ASPH is a key player in the malignant transformation of solid tumors through enhancing cell proliferation, migration, and invasion. Furthermore, ASPH promotes tumor growth through stimulation of angiogenesis and immunosuppression. These effects are mainly achieved via the activation of the Notch and SRC signaling pathways. ASPH expression is upregulated by growth factors and hypoxia in different human tumors, and its inactivation may have broad clinical effects. Moreover, ASPH expression is silenced in normal adult breast tissue, upregulated in malignancies in situ, and highly expressed in invasive ductal carcinoma [24].

Here we show that higher differential expression of ASPH correlated with tumor recurrence in ER + patients with breast cancer, including those treated with tamoxifen. These findings indicate that ASPH will serve as a predictor of recurrent triple-negative breast cancer or the *HER2*-amplified subtype as well as the ER + luminal A or B subtype. Thus, the pathway mediated by ASPH might not be affected by tamoxifen.

Tumor cells employ diverse oncogenically and environmentally driven metabolic pathways to satisfy the bioenergetics and biosynthetic requirements of rapid and sustained growth of tumor cells [25]. Glutamine supports the survival of cancers through its catalytic conversion to glutamate by glutaminase (GLS) in mitochondria [26, 27]. Glutamate and its metabolites further contribute to rate-limiting cellular metabolic pathways such as the tricarboxylic acid (TCA) cycle, redox, and amino acid synthesis. The survival of triple-negative primary breast cancers and the cognate breast cancer-derived cell lines in vitro is associated with differentially elevated levels of *GLS* mRNA that support high glutamine utilization and increased dependence on exogenous glutamine [28]. Here we show that higher differential expression of GLS correlated with tumor recurrence in ER + patients with breast cancer, including those treated with tamoxifen. These findings indicate that GLS will serve as a biomarker of recurrence of triple-negative

breast cancer as well as the ER + luminal A or B subtype. GLS may therefore represent a target of therapy designed to prevent cancer relapse, particularly after lymph nodes metastasis.

The predictive value of our gene expression profile with respect to breast cancer recurrence could be confirmed in larger number of lymph-bide-positive patients or verified in protein level. It might be a new direction that a large number of the same type of receptors as targets, rather than a single target, to develop drugs.

Conclusion

Sixteen genes encoding proteins with diverse functions and 73 genes encoding olfactory receptors were identified for the first time as potential biomarkers of breast cancer recurrence of patients with the ER + phenotype accompanied by lymph nodes metastasis before surgery. These genes and their products may therefore serve as targets of therapy designed to prevent cancer relapse, particularly after lymph nodes metastasis.

Abbreviation

formalin-fixed paraffin embedded (FFPE); estrogen receptor (ER); The Kyoto Encyclopedia of Genes and Genomes (KEGG); glutaminase (GLS); Aspartate beta-hydroxylase (ASPH);

Declarations

Ethical Approval and Consent to participate

The study was performed in accordance with the guideline laid down in the declaration of Helsinki. All patients gave their informed consent to participate in this study. The Medical Ethics Committee, Shanghai Institute of Planned Parenthood Research, approved the protocols for RNA extraction, microarray hybridization, and data analysis (approval number PJ2019-18).

Consent for publication

We confirm that this manuscript has not been published elsewhere and is not under consideration in whole or in part by another journal. All authors have approved the manuscript and agree with submission to *Breast Cancer Research*.

Availability of supporting data

The microarray data were deposited in the Gene Expression Omnibus (GEO) database (accession code GSE183231).

Competing interests

The authors declare no competing interests.

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Authors' contributions

ZSJ designed the study and helped prepare the manuscript. LY and SSM performed the data analysis. FZ and TX performed the experiments. EL wrote the manuscript.

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Figures

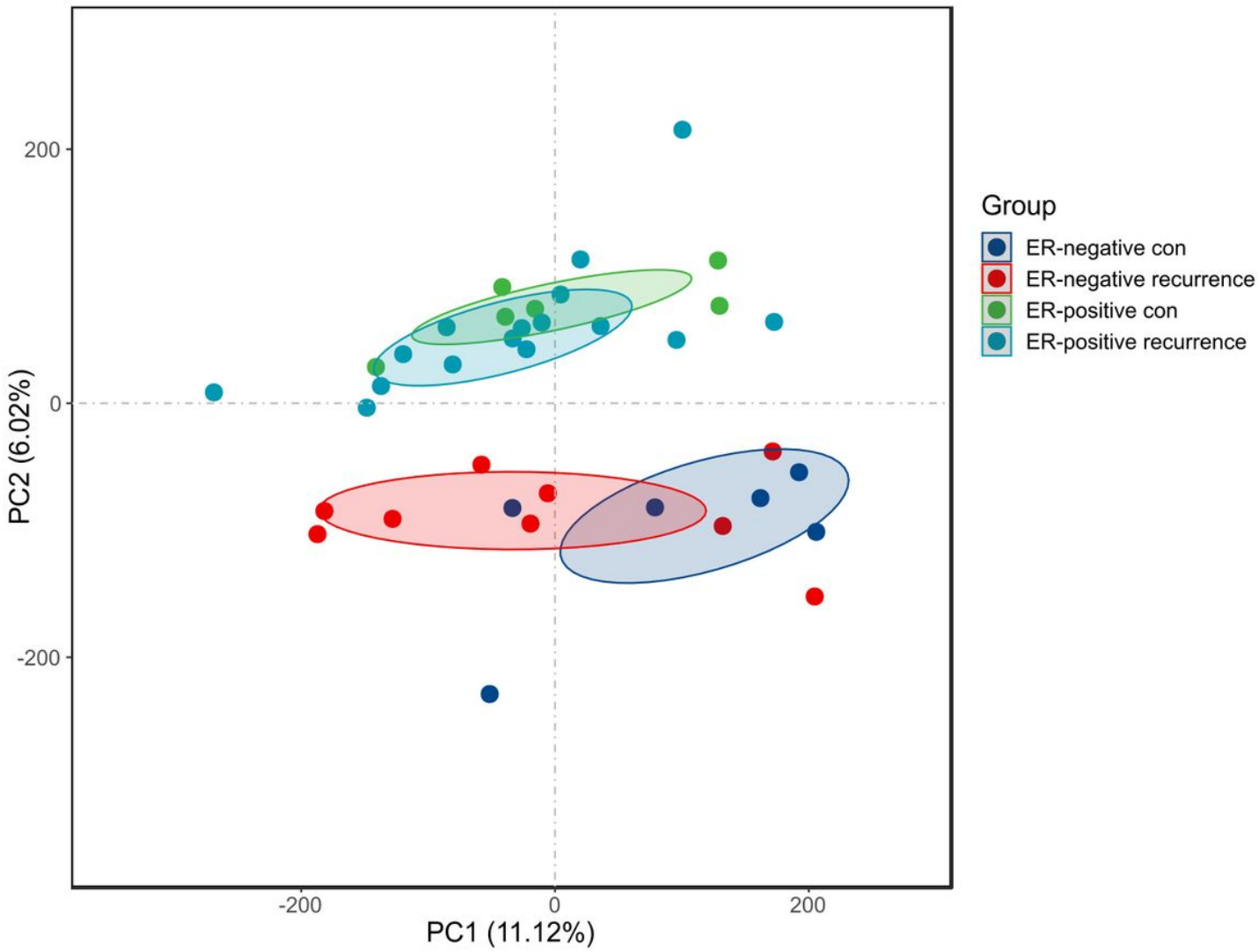

Figure 1

Principal co-ordinates analysis (PCoA) based on gene expression.

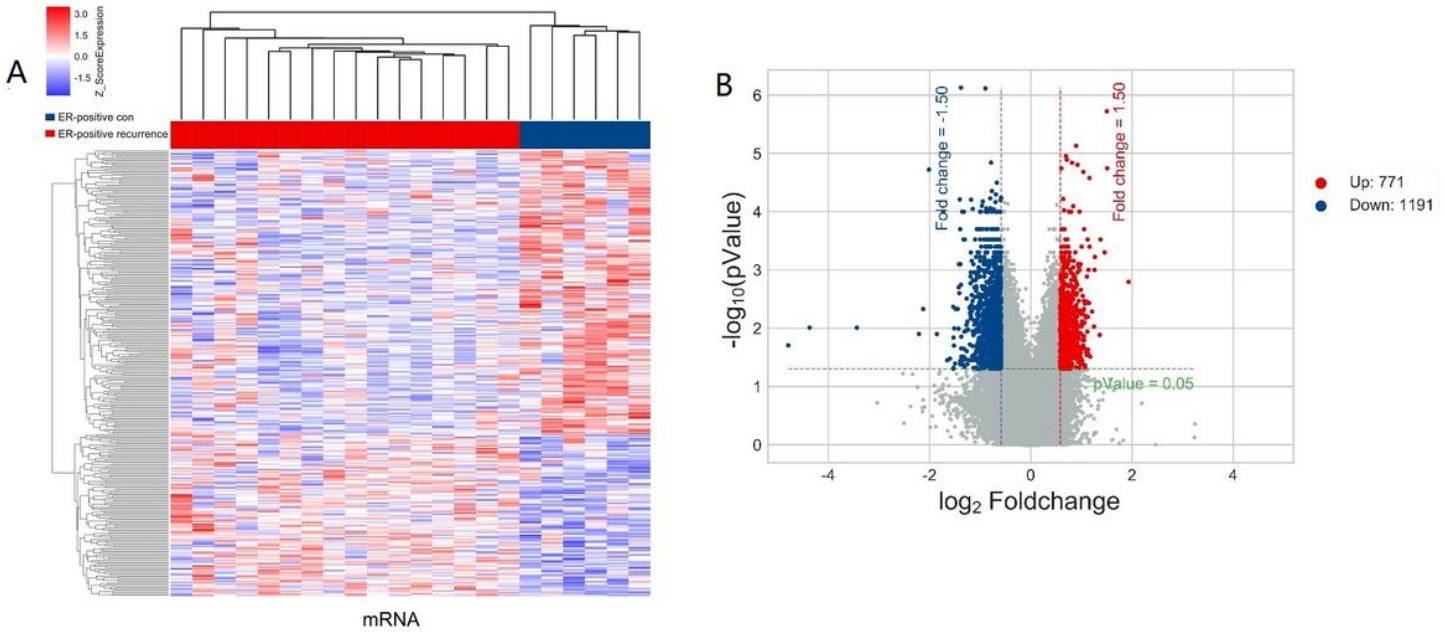

Figure 2

Identification of differentially expressed genes between recurrence and non-recurrence samples of ER+ breast cancer patients. A. The expression heatmap of differentially expressed genes. B. The volcano plot of differentially expressed genes.

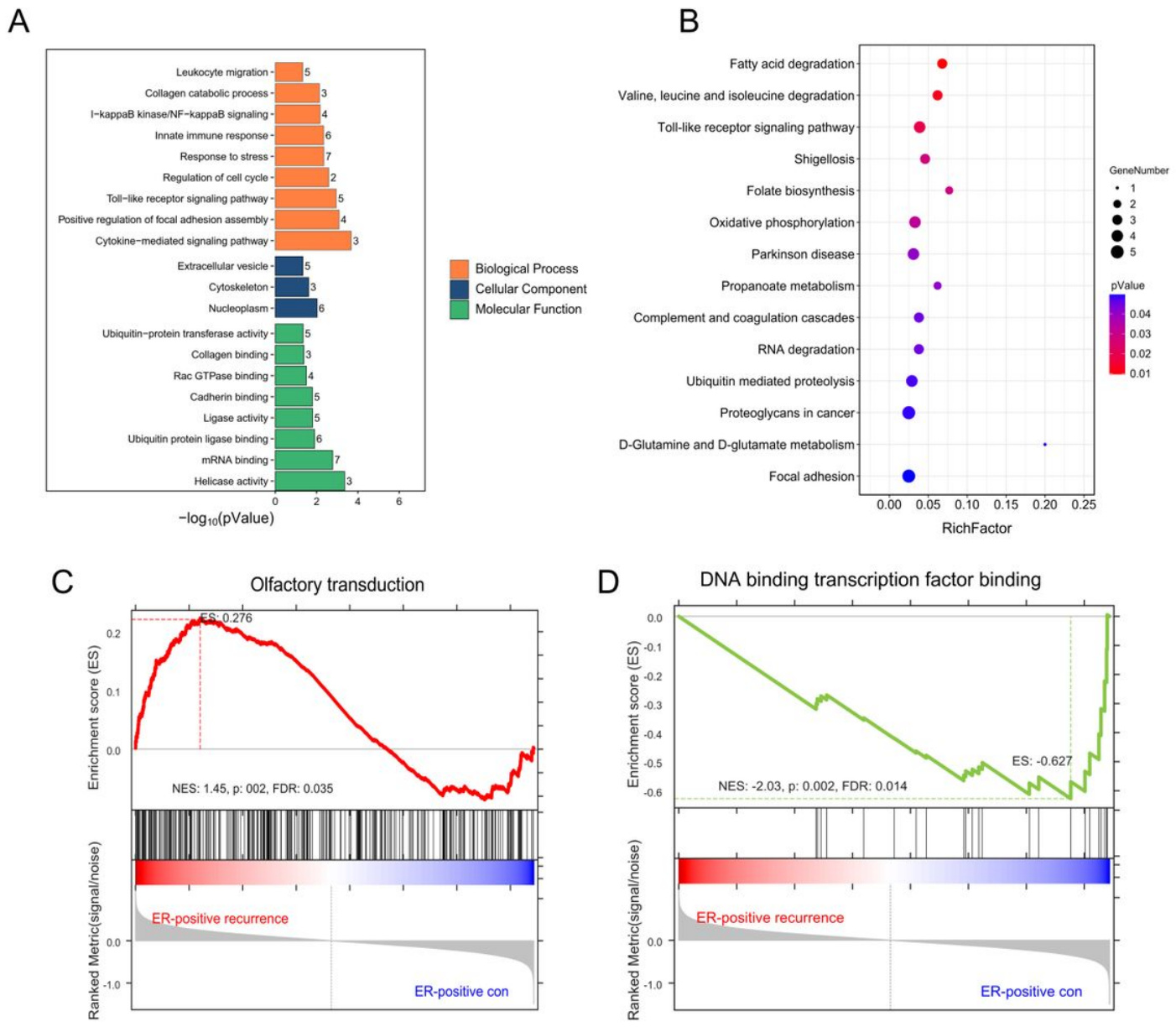

Figure 3

Functional enrichment analysis of differentially expressed genes between recurrence and non-recurrence samples of ER+ breast cancer patients. A. Top 20 of enriched GO terms. B. Enriched KEGG pathways. C. Enriched KEGG pathway of Olfactory transduction found by GSEA. D. Enriched GO term of DNA binding transcription factor binding found by GSEA.

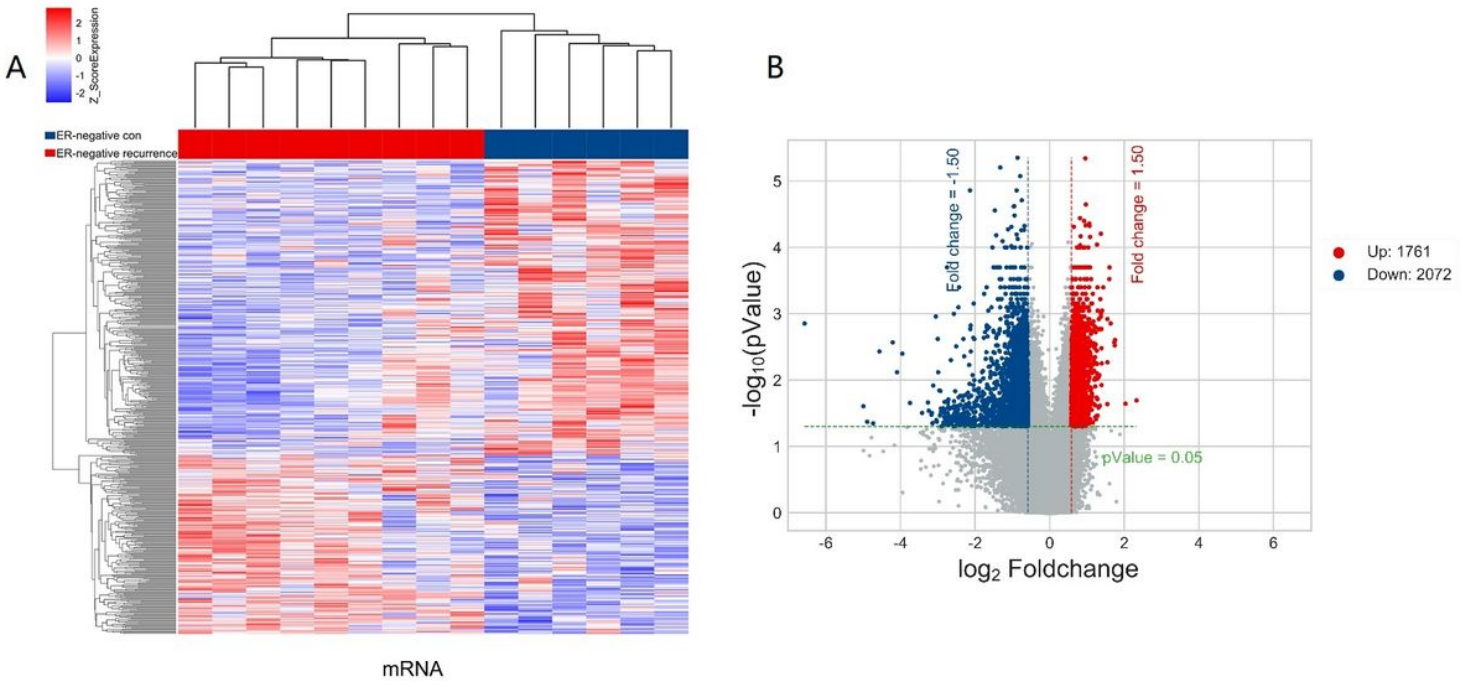

Figure 4

Identification of differentially expressed genes between recurrence and non-recurrence samples of ER- breast cancer patients. A. The expression heatmap of differentially expressed genes. B. The volcano plot of differentially expressed genes.

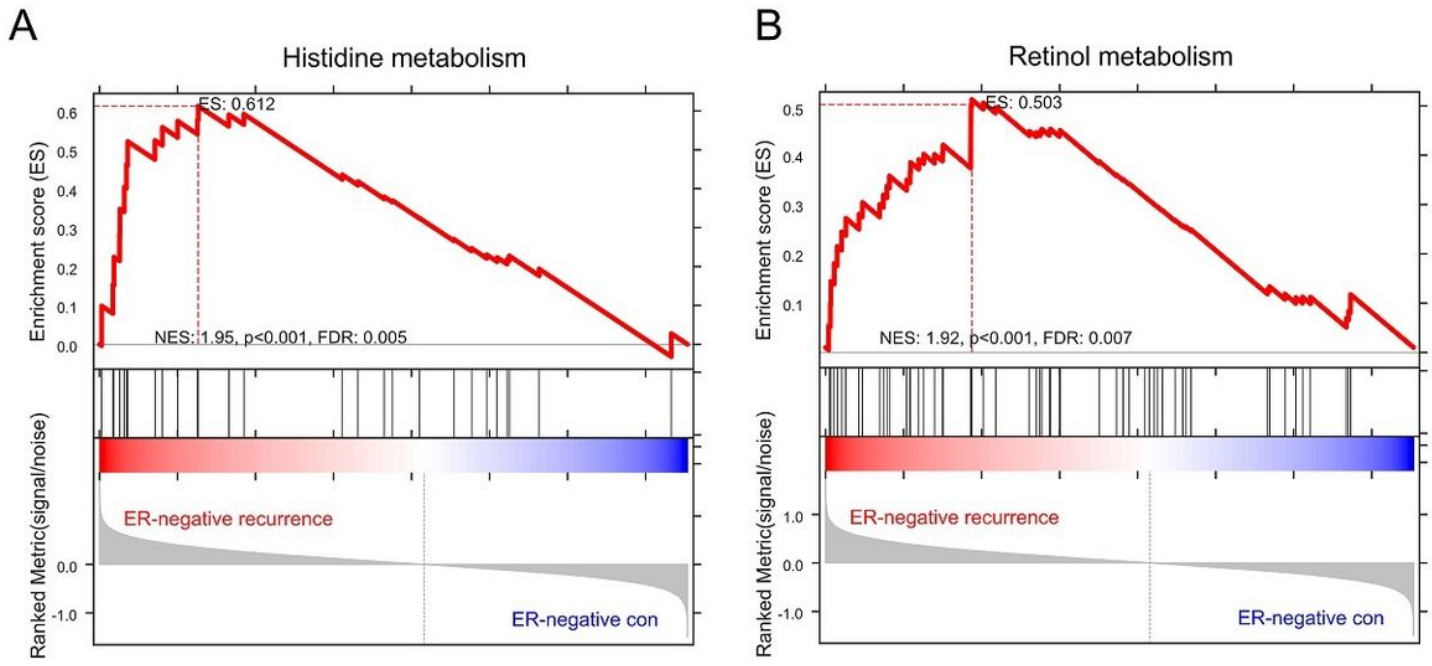

Figure 5

Functional enrichment analysis of differentially expressed genes between recurrence and non-recurrence samples of ER- breast cancer patients. A. Enriched KEGG pathway of Histidine metabolism found by GSEA. B. Enriched KEGG pathway of Retinol metabolism found by GSEA.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfilesv1.xls