ORIGINAL RESEARCH

Profiles of differentially expressed circRNAs in esophageal and breast cancer

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Introduction: Circular RNAs (circRNAs) function as efficient microRNA sponges with gene-regulatory potential and are promising cancer biomarkers. In this study, we used the Arraystar Human circRNA Array to construct a genome-wide circRNA profile of esophageal squamous cell cancer (ESCC) and breast cancer (BC).

Patients and methods: Expression levels between cancer lesions and adjacent normal-appearing tissues were compared. We observed 469 upregulated circRNAs and 275 downregulated circRNAs in ESCC. Hsa_circRNA_103670 was upregulated 20.3-fold, while hsa_circRNA_030162 was downregulated 12.1-fold. For BC, 715 circRNAs were upregulated, and 440 circRNAs were downregulated. Hsa_circRNA_005230 was upregulated 12.2-fold, while hsa_circRNA_406225 was downregulated 12.4-fold.

Results: When we set the criteria as fold change in expression ≥2 between cancer and adjacent normal-appearing tissue with a P-value <0.01, there were 22 common circRNAs (11 upregulated and 11 downregulated) in relation to both ESCC and BC. Gene ontology and the Kyoto encyclopedia of genes and genomes analyses showed that these circRNAs were involved in the tumorigenesis of human cancers.

Conclusion: Our study revealed that circRNAs are promising candidates as valuable biomarkers for ESCC and BC, although relevant research is still in its infancy and the functional role of specific circRNAs in tumorigenesis is just starting to be elucidated.

Keywords: circRNA, noncoding RNA, esophageal squamous cell carcinoma, breast cancer, biomarker

Introduction

Cancer is the leading cause of death in developed countries, and its prevalence is increasing in developing countries as well, which imposes a heavy burden to society at large. According to a GLOBOCAN report, there were an estimated 14.1 million new cancer cases and 8.2 million cancer-related deaths in 2012 worldwide.[1,2] Esophageal cancer ranks as the eighth most common cancer worldwide and ranks sixth in cancer-causing deaths. Histologically, the majority of esophageal cancers are divided into squamous cell carcinoma and adenocarcinoma. The high-risk areas include northern Iran, southern Russia, central Asian countries, and northern China, where esophageal squamous cell cancer (ESCC) accounts for 90% of all esophageal cancer cases.[3] The prognosis of ESCC is poor, and patients have only 15%–25% of 5-year survival rates after diagnosis.[4–6]

The poor prognosis and rising incidence of esophageal cancer have highlighted the need for improved detection and prediction methods that are essential prior to treatment.[7] Current clinically approved surveillance practices highly depend on expensive,

invasive, and sampling-error-prone endoscopic procedures.[8] Therefore, there is a great demand to establish reliable biomarkers that could identify patients at higher risks of neoplastic progression who would hence greatly benefit from further monitoring and/or intervention. Emerging molecular tools promise to extend the diagnostic research of the endoscopist and open doors to population screening for ESCC.[9] An increasing evidence has shown that noncoding RNAs, such as microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circular RNAs (circRNAs), play an important role in the development and progression of multiple human cancers and could be used as prognostic factors and therapeutic targets for esophageal cancer.[10]

CircRNAs are a class of noncoding RNA molecules that lack 5′3′ ends and a poly A tail, covalently forming closed continuous loops.[11,12] In general, circRNAs are stable molecules, and some have functioned as efficient miRNA sponges with gene-regulatory ability.[13] CircRNAs, with their distinctive characteristics, have superior potential to serve as novel markers for human diseases. The list of endogenous circRNAs involved in cancer continues to grow; however, the functional relevance of the majority of endogenous circRNAs has yet to be discovered.[13]

In one of our previous studies, we have provided evidence that circRNAs are differentially expressed in breast cancer (BC) and play an important role in carcinogenesis because they participate in cancer-related pathways and sequester miRNAs.[14] However, whether circRNAs are sensitive and specific biomarkers of ESCC and BC remains largely unknown. In this study, we used an Arraystar human circRNA array (Arraystar Inc., Rockvile, MD, USA) to construct a genome-wide circRNA profile of ESCC compared with altered expression in BC, with the aim of exploring the potential functions of these circRNAs as diagnostic biomarkers.

Patients and methods
Ethics statement
This study was approved by the Institutional Review Board of Nanjing Medical University, China. Written informed consent was obtained from all participants included in the study.

Patients
Patients with ESCC were enrolled from the First People's Hospital of Yancheng in October 2016. Tissues in the cancer lesion and adjacent normal-appearing esophagus were collected from patients who underwent surgical resection and met the following criteria: 1) a pathologic diagnosis of ESCC; 2) no previous history of cancer; 3) HIV negative; and 4) no

history of radiotherapy or chemotherapy before specimen collection. The recruitment process of patients with BC was detailed in a previously published paper.[14] Tissue samples were placed in RNA storage solution (Shanghai Biotechnology Corporation, Shanghai, China) and stored at −80°C until use. Tumor stages were determined according to the tumor-node-metastasis (TNM) staging criteria.[15]

CircRNA microarray
The RNA was isolated with an RNeasy mini kit (Qiagen, Hilden, Germany) and analyzed using an 8*15K Arraystar human circRNA microarray V2 (Catalog No: AS-CR-H-V2.0). Sample preparation and array hybridization followed the manufacturer's protocol.

Bioinformatics and data analysis
We performed gene ontology (GO) analysis (http://www.geneontology.org)[16] to construct meaningful annotation of genes in any organism covering domains of biological processes, cellular components, and molecular functions. The log10(P-value) denotes the significance of GO term enrichment correlated to the genes producing differentially expressed circRNAs. Kyoto encyclopedia of genes and genomes (KEGG) analysis (http://www.genome.jp/kegg)[17] was carried out to confirm the pathway clusters covering molecular interaction and reaction networks in genes producing differentially expressed circRNAs.

Statistical analysis
The fold change in circRNA expression was calculated by comparing expression levels between cancer lesions and control tissues. Student's t-test was used to estimate the significance of the difference between the two groups. CircRNAs with fold change ≥2 and $P<0.05$ were considered to be statistically significant. We used the filter criteria of fold change ≥2 and $P<0.01$ to screen for common differentially expressed circRNAs shared by both ESCC and BC. The false discovery rate was used to adjust for P-values in microarray analysis. Agilent feature extraction software (version 11.0.1.1, Agilent, Santa Clara, CA, USA) was used to analyze the acquired array images. R software version 3.3.1 (https://www.r-project.org/)[18] was used to perform quantile normalization and for GO and KEGG analysis.

Results
Differentially expressed circRNAs
Seven paired ESCC samples and four paired BC samples were collected for the Arraystar human circRNA array. The

characteristics of the patients are shown in Tables S1 and S2. When we set the criteria as fold change ≥2.0 and *P*<0.05 between cancer lesions and adjacent normal-appearing tissues, there were 744 differentially expressed circRNAs in ESCC and 1155 differentially expressed circRNAs in BC. For ESCC, 469 circRNAs were upregulated, and 275 circRNAs were downregulated. The top 10 upregulated and top 10 downregulated circRNAs for ESCC are listed in Table 1. Hsa_circRNA_103670 was upregulated 20.3-fold, while hsa_circRNA_030162 was downregulated 12.1-fold. For BC, 715 circRNAs were upregulated, and 440 circRNAs were downregulated. The top 10 upregulated and top 10 downregulated circRNAs of BC are listed in Table 2. Hsa_circRNA_005230 was upregulated 12.2-fold, while hsa_circRNA_406225 was downregulated 12.4-fold.

Figure 1 illustrates the hierarchical clustering analysis showing the distinct circRNA expression profiling in ESCC (Figure 1A) and BC (Figure 1B). Figure 2 shows the scatter plots demonstrating the heterogeneity of circRNA expression of ESCC cancer lesions (Figure 2A) and BC cancer lesions (Figure 2B) with their adjacent normal-appearing tissues. The expression of the circRNAs above the top reference line and below the bottom reference line changed by >2-fold.

Volcano plots were used to visualize the significantly differentially expressed circRNAs for ESCC (Figure 3A) and BC (Figure 3B).

When we set the criteria as the fold change in expression ≥2 between cancer and adjacent normal-appearing tissue and *P*<0.01, there were 22 common circRNAs in relation to both ESCC and BC. Among them, 11 were upregulated and 11 were downregulated (Table 3).

GO enrichment and KEGG analysis

We further used GO analysis to explore the roles of these dysregulated circRNAs. The top 10 upregulated circRNAs in ESCC were related to positive regulation of cellular processes, biological processes, and macromolecule metabolic processes (Figure S1A), whereas the top 10 downregulated circRNAs in ESCC were related to positive regulation of cellular metabolic processes, RNA metabolic processes, and cellular processes (Figure S1B). In BC, the top 10 upregulated circRNAs were associated with negative regulation of cellular processes, biological processes, and macromolecule metabolic processes (Figure S1C), while the top 10 downregulated circRNAs were associated with the regulation of macromolecule metabolic processes, metabolic processes,

Table 1 The top 10 upregulated and top 10 downregulated circRNAs for ESCC

Upregulated circRNAs	*P*-value	Fold change	Downregulated circRNAs	*P*-value	Fold change
hsa_circRNA_103670	0.021	20.321	hsa_circRNA_030162	0.014	12.071
hsa_circRNA_004390	0.004	7.870	hsa_circRNA_001729	<0.001	9.382
hsa_circRNA_104172	0.004	7.686	hsa_circRNA_087212	0.044	9.087
hsa_circRNA_404013	0.014	7.522	hsa_circRNA_037767	0.015	8.543
hsa_circRNA_001937	0.015	6.517	hsa_circRNA_000367	<0.001	8.223
hsa_circRNA_100872	0.006	6.255	hsa_circRNA_102459	0.012	7.413
hsa_circRNA_102854	<0.001	5.763	hsa_circRNA_001640	0.001	7.021
hsa_circRNA_406826	0.004	5.552	hsa_circRNA_020068	0.024	6.912
hsa_circRNA_013058	0.004	5.249	hsa_circRNA_004183	<0.001	6.724
hsa_circRNA_004662	<0.001	5.127	hsa_circRNA_400850	0.001	6.529

Abbreviations: circRNA, circular RNA; ESCC, esophageal squamous cell cancer.

Table 2 The top 10 upregulated and top 10 downregulated circRNAs for BC

Upregulated circRNAs	*P*-value	Fold change	Downregulated circRNAs	*P*-value	Fold change
hsa_circRNA_005230	0.045	12.198	hsa_circRNA_406225	0.032	12.406
hsa_circRNA_103254	0.006	8.155	hsa_circRNA_404724	0.021	12.154
hsa_circRNA_103552	0.006	7.201	hsa_circRNA_406697	0.008	9.130
hsa_circRNA_101981	0.003	6.762	hsa_circRNA_002873	0.013	8.908
hsa_circRNA_063763	0.005	6.725	hsa_circRNA_008842	0.043	8.860
hsa_circRNA_103253	0.006	6.467	hsa_circRNA_047418	0.018	7.477
hsa_circRNA_103902	0.010	5.929	hsa_circRNA_401033	0.034	7.371
hsa_circRNA_002908	0.003	5.895	hsa_circRNA_001288	0.022	7.321
hsa_circRNA_102736	0.012	5.723	hsa_circRNA_104858	0.025	6.891
hsa_circRNA_104327	0.020	5.682	hsa_circRNA_001838	0.037	6.875

Abbreviations: circRNA, circular RNA; BC, breast cancer.
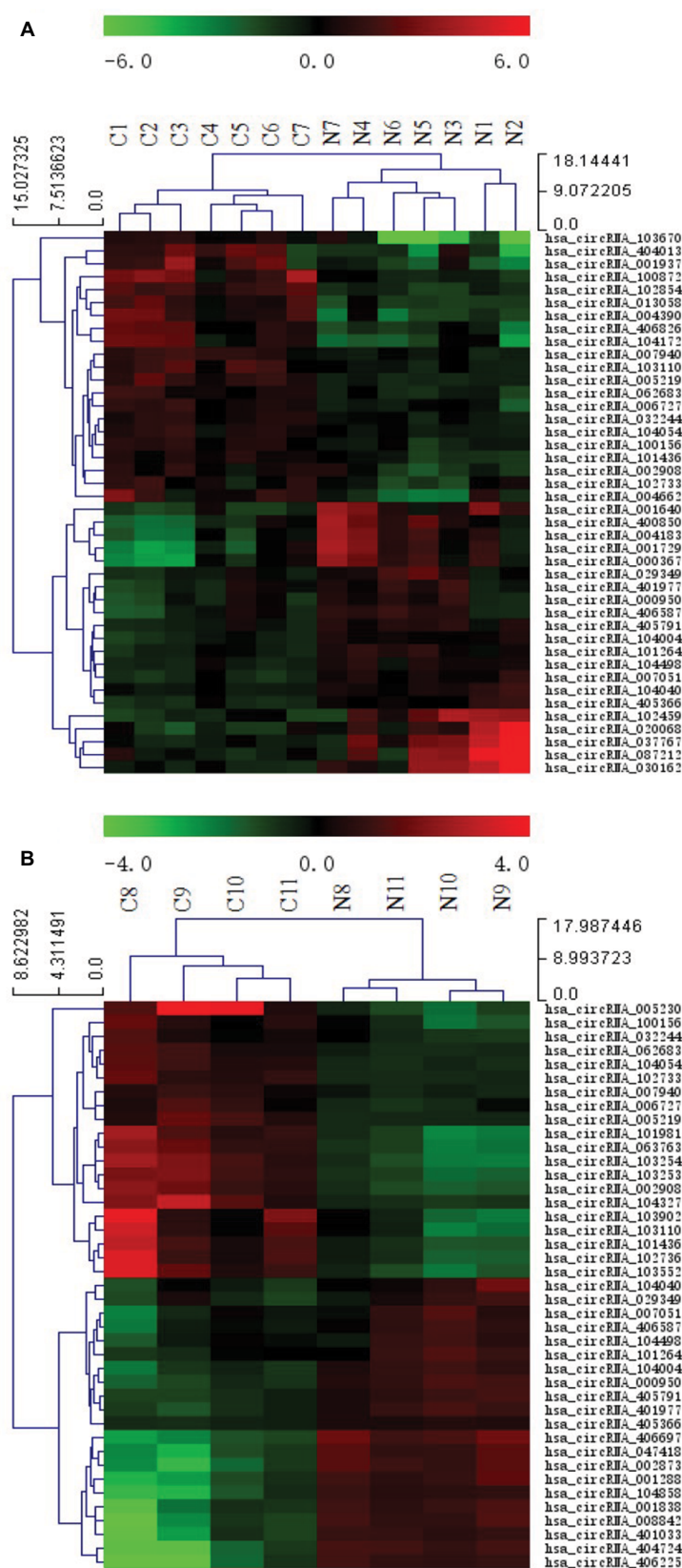
Figure 1 Heat map showing the expression profiles of circRNAs in ESCC (**A**) and BC (**B**).
Notes: The expression values are represented by the color scale. The intensity increases from green (relatively lower expression) to red (relatively higher expression). Each column represents one tissue sample, and each row represents a single circRNA. C1–C7 represent seven ESCC specimens collected from ESCC patients. N1–N7 represent paired adjacent normal-appearing tissues. C8–C11 represent four BC specimens collected from BC patients. N8–N11 represent paired adjacent normal-appearing tissues.
Abbreviations: ESCC, esophageal squamous cell cancer; BC, breast cancer.

A

B
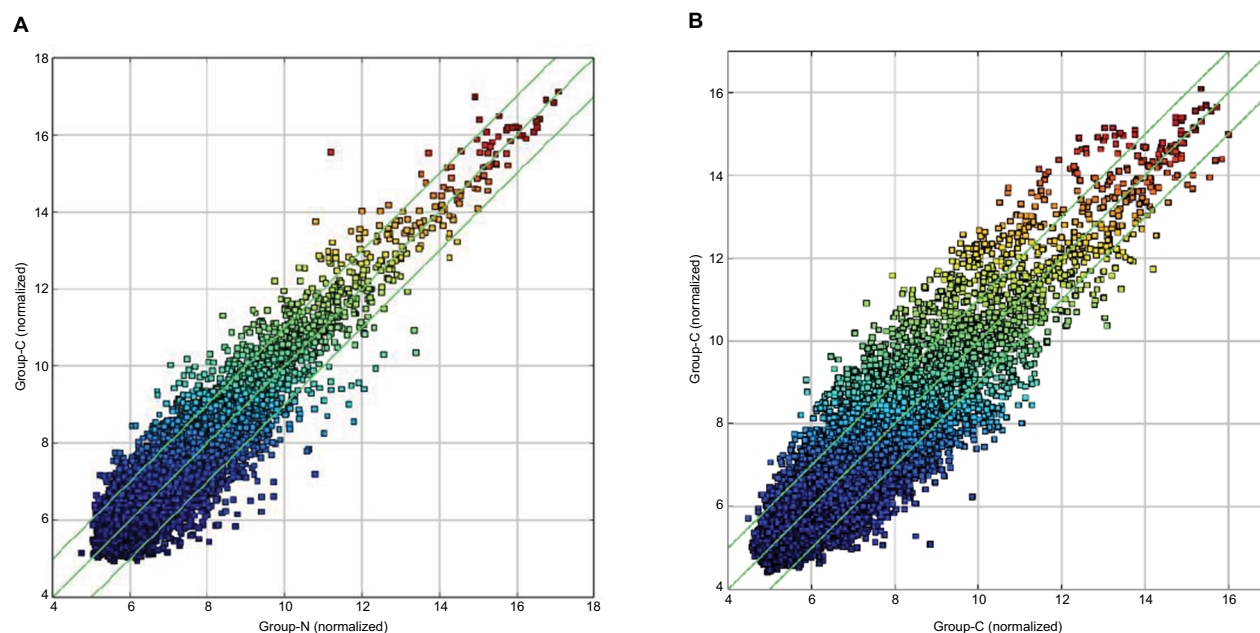
Figure 2 Scatter plots demonstrating the heterogeneity of ESCC lesions (**A**) and BC lesions (**B**) with their adjacent normal-appearing tissues.
Notes: The values of the X and Y axes represent the averaged normalized signal values of the group (log2-scaled). The green line stands for 2-fold changes. The expression of the circRNAs above the top green line and below the bottom green line indicate changes by >2-fold between the two groups of samples.
Abbreviations: ESCC, esophageal squamous cell cancer; BC, breast cancer.

A

B
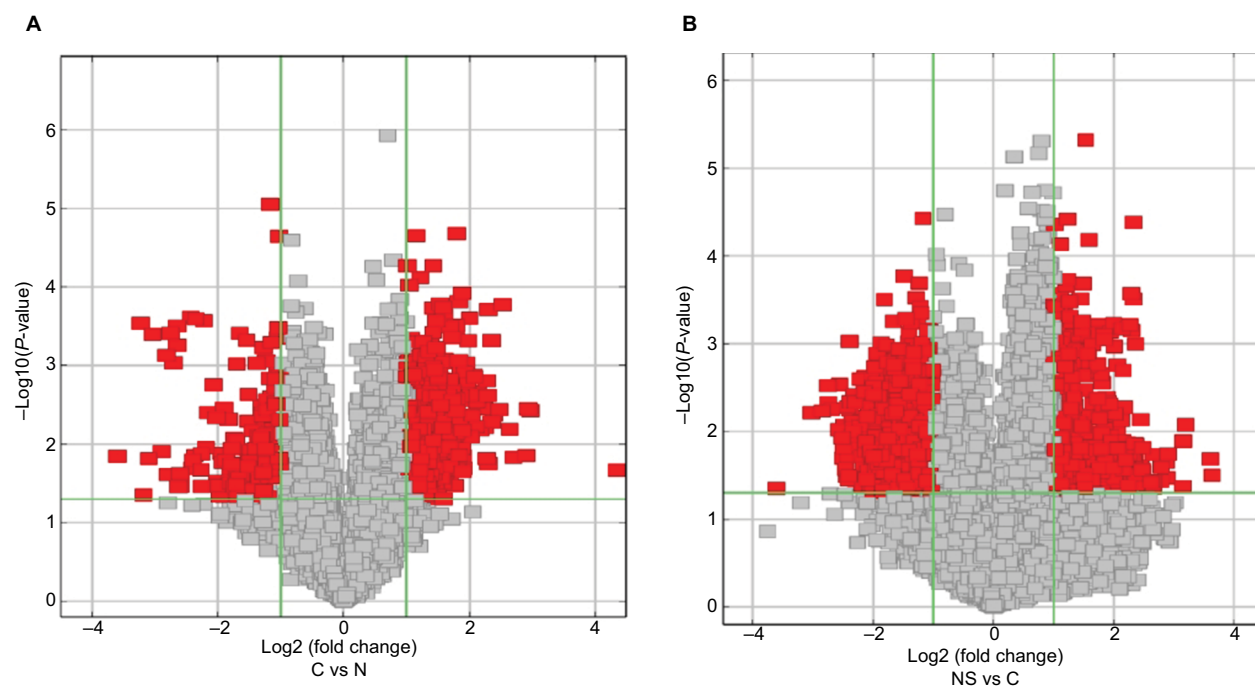
Figure 3 Volcano plots visualizing differential expression of ESCC lesions (**A**) and BC lesions (**B**) with adjacent normal tissues.
Notes: The vertical lines correspond to 2.0-fold up- and downregulation (log2 ratio), and the horizontal line represents a P-value of 0.05. The red points in the plot represent the differentially expressed circRNAs with statistical significance (fold change >2 and P<0.05), the gray points represent the remaining circRNAs (fold change <2 or P>0.05).
Abbreviations: ESCC, esophageal squamous cell cancer; BC, breast cancer.

and primary metabolic processes (Figure S1D). We further performed GO analysis on 22 dysregulated circRNAs shared in both ESCC and BC. Our data revealed that 11 shared upregulated circRNAs were involved in the regulation of signaling, cellular processes, and biological processes (Figure S1E), while 11 shared downregulated circRNAs were involved in the regulation of signaling and developmental processes (Figure S1F).

Table 3 Common circRNAs in relation to both ESCC and BC

CircRNAs	ESCC		BC	
	P-value	Fold change	*P*-value	Fold change
Upregulated				
hsa_circRNA_002908	0.008	2.447	0.003	5.895
hsa_circRNA_005219	0.001	3.556	0.009	2.945
hsa_circRNA_006727	0.009	2.51	0.005	2.301
hsa_circRNA_007940	0.001	2.688	0.009	2.068
hsa_circRNA_032244	0.002	2.029	0.004	2.073
hsa_circRNA_062683	0.005	2.741	0.006	2.568
hsa_circRNA_100156	0.005	2.26	0.001	3.209
hsa_circRNA_101436	0.009	2.42	0.008	5.084
hsa_circRNA_102733	0.001	2.193	0.008	2.930
hsa_circRNA_103110	0.006	2.987	0.005	5.189
hsa_circRNA_104054	0.007	2.019	0.008	2.887
Downregulated				
hsa_circRNA_000950	0.008	2.095	0.001	3.847
hsa_circRNA_007051	0.001	2.592	0.005	2.555
hsa_circRNA_029349	0.001	2.409	0.009	2.158
hsa_circRNA_101264	<0.001	2.245	0.006	2.313
hsa_circRNA_104004	0.004	2.154	0.002	3.455
hsa_circRNA_104040	0.004	2.024	0.005	2.473
hsa_circRNA_104498	<0.001	2.041	<0.001	2.028
hsa_circRNA_401977	0.005	2.074	0.006	3.263
hsa_circRNA_405366	0.008	2.463	0.002	2.019
hsa_circRNA_405791	0.003	2.216	0.007	3.039
hsa_circRNA_406587	0.001	2.347	0.007	2.564

Abbreviations: circRNA, circular RNA; ESCC, esophageal squamous cell cancer; BC, breast cancer.

In KEGG pathway analysis, for the top 10 upregulated circRNAs in ESCC, HTLV-I infection, cancer, and cGMP-PKG signaling were the top three pathways (Figure S2A). For the top 10 downregulated circRNAs in ESCC, Wnt signaling, ubiquitin-mediated proteolysis, and HTLV-I infection were the top three pathways (Figure S2B). Moreover, the top three KEGG pathways for the top 10 upregulated circRNAs in BC were MAPK signaling, neurotrophin signaling, and ubiquitin-mediated proteolysis (Figure S2C), and the top three KEGG pathways for the top 10 downregulated circRNAs in BC were MAPK signaling, synaptic vesicle cycle, and hippo signaling pathway-multiple species (Figure S2D). For the 11 upregulated circRNAs shared in ESCC and BC, the three most enriched pathways in the KEGG analysis were endocytosis, thyroid hormone signaling pathway, and proteoglycans in cancer (Figure S2E). The three most enriched pathways for the 11 downregulated circRNAs shared in ESCC and BC were proteoglycans in cancer, endocytosis, and pathways in cancer (Figure S2F).

Discussion

CircRNAs are RNA molecules in which a covalent linkage typically contains the exon sequences and a splice between an upstream 3′ splice site and a downstream 5′ splice site.[19,20] CircRNAs have been hypothesized to function as miRNA sponges to offset the impact of miRNAs. In recent years, more roles of circRNAs such as sequestering proteins or regulating transcription have been discovered.[21] Studies have shown that abnormal circRNAs are involved in tumorigenesis and disease progression.[22–25] However, whether they are general cancer biomarkers or cancer specific biomarkers is not clear. To distinguish the differentially expressed circRNAs between ESCC and BC, we performed a comparative study.

For ESCC, hsa_circRNA_103670 was the most upregulated circRNA and aligned with *CNOT6L* (CCR4-NOT transcription complex subunit 6 like, Gene ID: 246175), which is a main deadenylase complex regulating gene expression in eukaryotes. The most downregulated was hsa_circRNA_030162. This circRNA derives from the gene *TPT1* (tumor protein translationally controlled 1, Gene ID: 7178), also called translationally controlled tumor protein (TCTP),[26,27] which encodes a cell growth-associated protein and plays an important role in the development of various organisms. *TPT1* has recently been identified as related to human skin squamous cell carcinoma and is targeted by miRNA-216b-5p in pancreatic cancer.[28,29]

For BC, the top upregulated circRNA was hsa_circRNA_005230. It was spliced from the gene *DNM3OS* (*DNM3* opposite strand/antisense RNA, Gene ID: 10062831), which produces a noncoding RNA (ncRNA) that is involved in the formation of miR-199a2 and miR-214.[30] These miRNAs have been reported to be downregulated in hepatocellular cancer.[31] The top downregulated circRNA was hsa_circRNA_406225. It aligned with the gene *TAMM41* (*TAM41* mitochondrial translocator assembly and maintenance homolog, Gene ID: 132001), which is a homolog of mitochondrial translocator assembly and maintenance protein.[32,33]

We observed 22 dysregulated circRNAs shared by both ESCC and BC. Among them, hsa_circRNA_002908 (hsa_circ_0002908) was found to be upregulated in peripheral blood mononuclear cells of tuberculosis patients compared with those of paired healthy controls and may be an alternative biomarker for pulmonary tuberculosis.[34] Hsa_circRNA_101436 (hsa_circ_0000567) can inhibit colorectal tumor growth and was upregulated in gefitinib-acquired resistant non-small-cell lung cancer cells.[35,36] Hsa_circRNA_103110 (hsa_circ_0004771) has been reported to be significantly expressed in BC14 and papillary thyroid carcinoma.[37] Hsa_circRNA_000950 (hsa_circ_0001525) and hsa_circRNA_104040 (hsa_circ_0075410) were listed

in the top 10 downregulated circRNAs from a microarray on cutaneous squamous cell carcinoma.[38] The expression of hsa_circRNA_401977 (hsa_circ_0000567) was found to be downregulated in early stage lung adenocarcinoma tissues and cell lines.[39] These findings provided evidence of the role of circRNAs in tumorigenesis.

KEGG analysis indicated that differentially expressed circRNAs were associated with multiple cancers. Many pathways are related to cancer-related functions. For example, the Wnt pathway has vital roles in the prognosis of non-small-cell lung cancer and may have future clinical value.[40] The Hippo signaling pathway, consisting of the critical downstream effectors YAP/TAZ, contributes to the development of cancer by resulting in an overgrowth phenotype.[41] The MAPK signaling pathway is an essential process for *CD97* to promote gastric cancer cell proliferation and invasion.[42]

Our study has several limitations. First, the sample size is too small to make any reasonable conclusion. These results might not even be educational for further studies by other research groups. Differentially expressed circRNAs discovered in this study need further validation. Second, the paired *t*-test used in this study to compare circRNA expression between the groups may not have been appropriate for the microarray experiment. Other robust hyperparameter estimations need to be used to protect against hypervariable genes and improve the power to detect differential expression.[43,44] Third, the study on the role of circRNAs in human cancers is still in its infancy, and a common standard for reporting and naming circRNAs is lacking. Fourth, we used tissue samples to detect circRNA. More easily acquired and noninvasive clinical samples, such as blood, urine, or saliva, should be explored as sources of biomarkers in future research.

Conclusion
In conclusion, circRNAs are promising candidates as valuable biomarkers for ESCC and BC, although circRNA research is still in its infancy, and the functional role of circRNAs in tumorigenesis is just starting to be elucidated.

Acknowledgments

Author contributions
PS and JMW conceived, initiated, and led the study. PS, JS, JMW, and HX analyzed the data with input from all the authors. PS and JMW prepared the manuscript. All authors contributed to data analysis, drafted and revised the paper, and agreed to be accountable for all aspects of the work. All authors reviewed and approved the manuscript.

Disclosure
The authors report no conflicts of interest in this work.

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Supplementary materials

A

Up-regulated circRNAs in ESCC

B

Down-regulated circRNAs in ESCC

Figure S1 (*Continued*)

Up-regulated circRNAs in BC

Down-regulated circRNAs in BC

Figure S1 (*Continued*)

E

11 shared up-regulated circRNAs in ESCC and BC

F

11 shared down-regulated circRNAs in ESCC and BC

Figure S1 GO enrichment analysis for differentially expressed genes.

Notes: (**A**) GO annotation of the top 10 upregulated circRNAs in ESCC; (**B**) GO annotation of the top 10 downregulated circRNAs in ESCC; (**C**) GO annotation of the top 10 upregulated circRNAs in BC; (**D**) GO annotation of the top 10 downregulated circRNAs in BC; (**E**) GO annotation of 11 shared upregulated circRNAs in ESCC and BC; (**F**) GO annotation of 11 shared downregulated circRNAs in ESCC and BC.

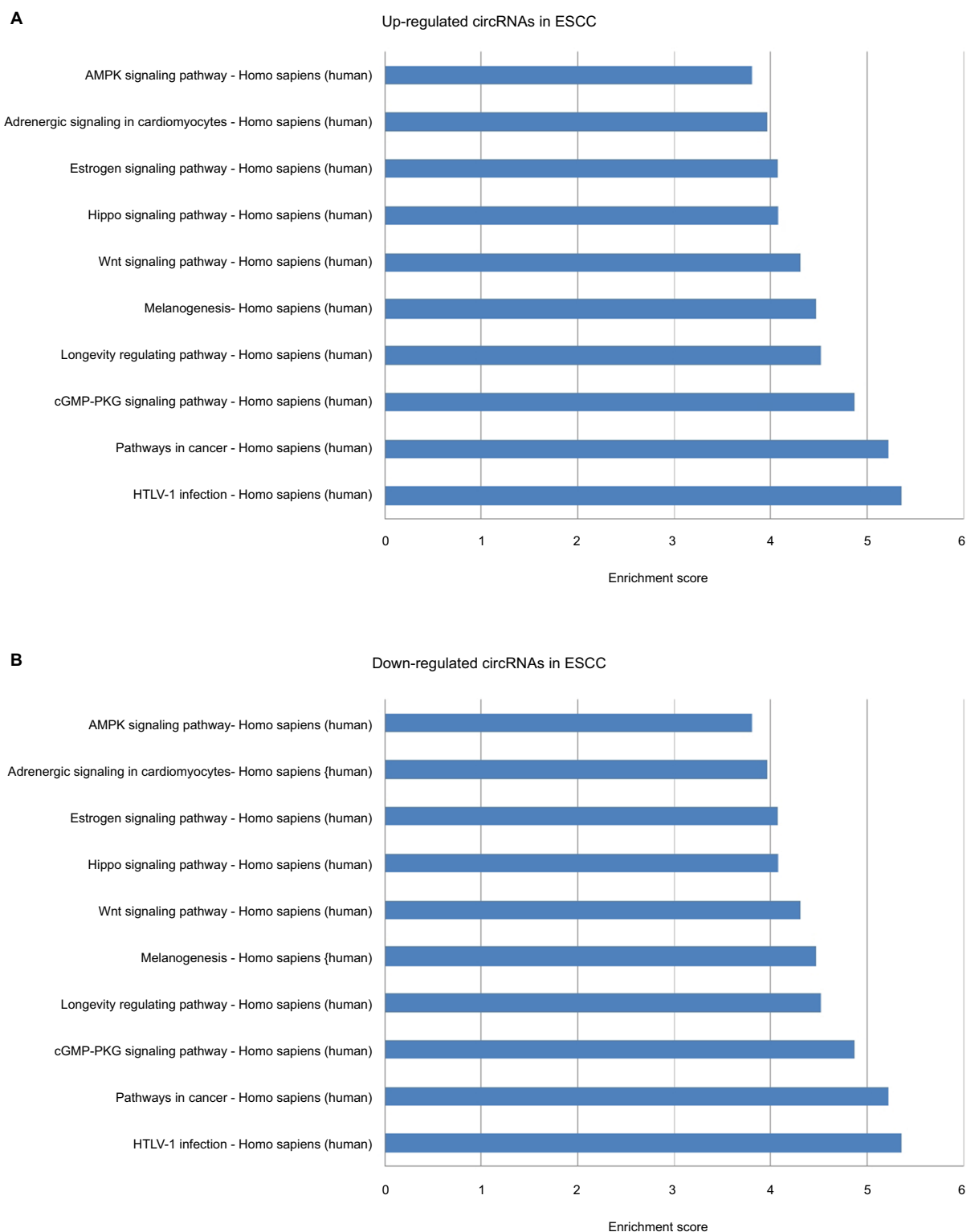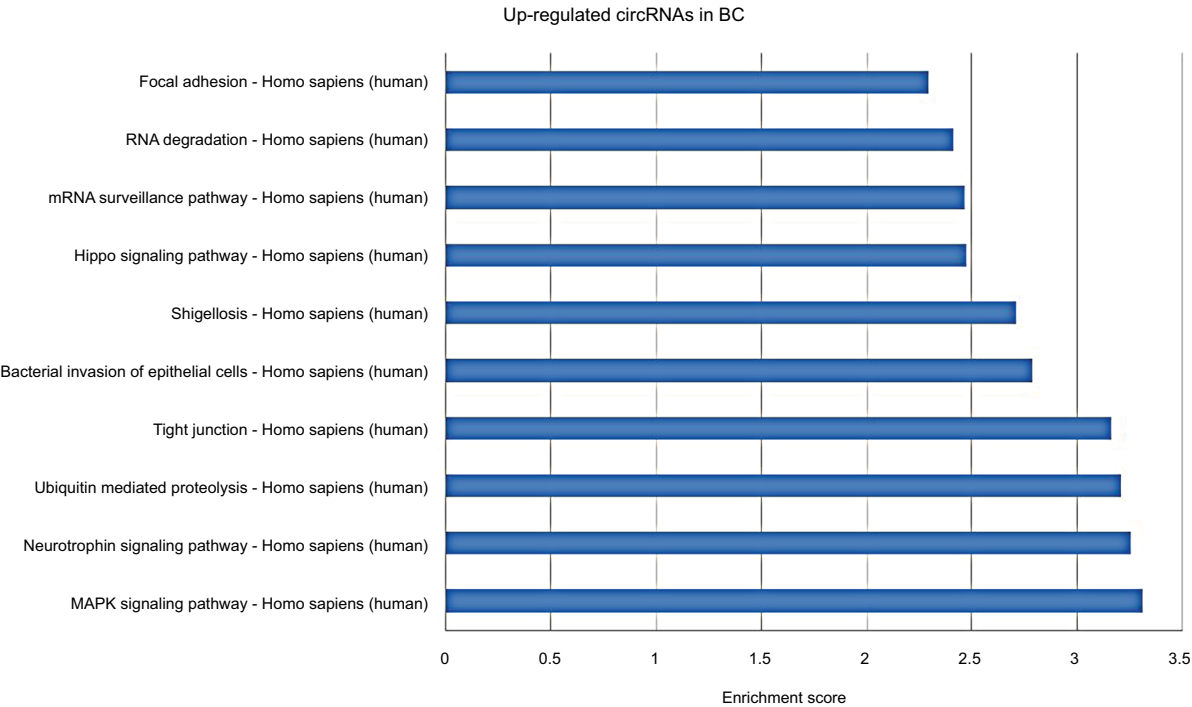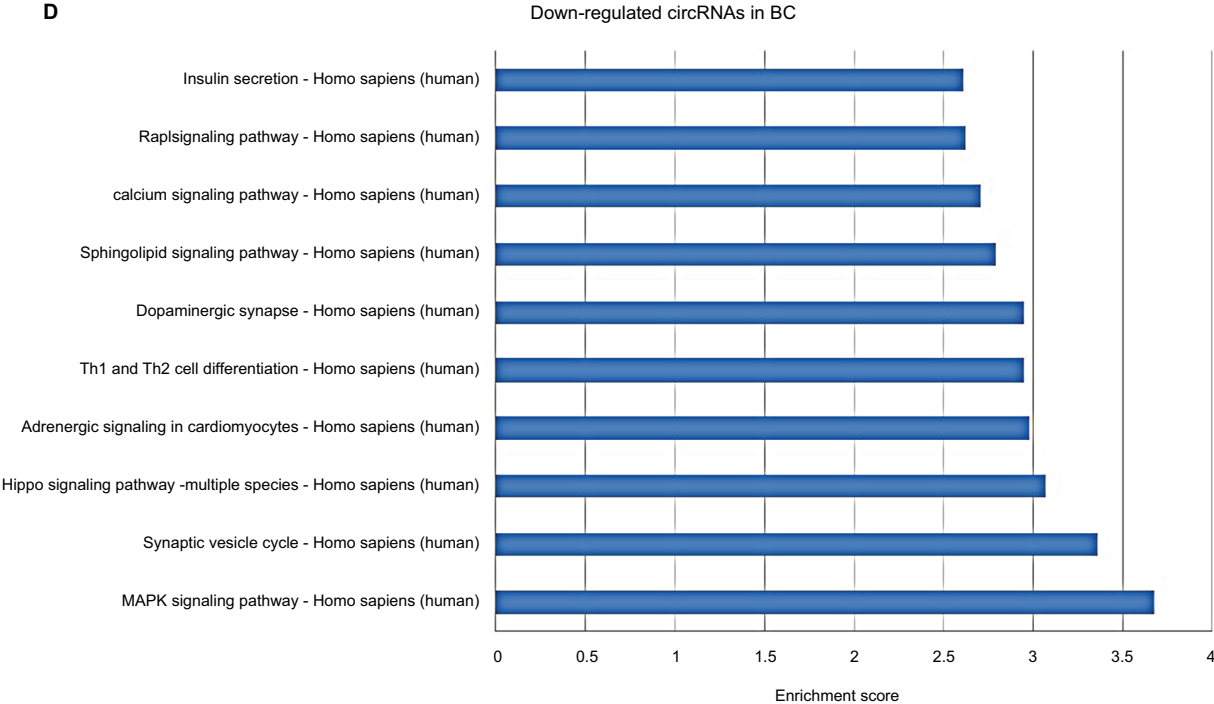**Abbreviations:** circRNA, circular RNA; ESCC, esophageal squamous cell cancer; BC, breast cancer.

A

Up-regulated circRNAs in ESCC

B

Down-regulated circRNAs in ESCC

Figure S2 (*Continued*)

C

Up-regulated circRNAs in BC

D

Down-regulated circRNAs in BC

Figure S2 (*Continued*)

E

11 shared up-regulated circRNAs in BC
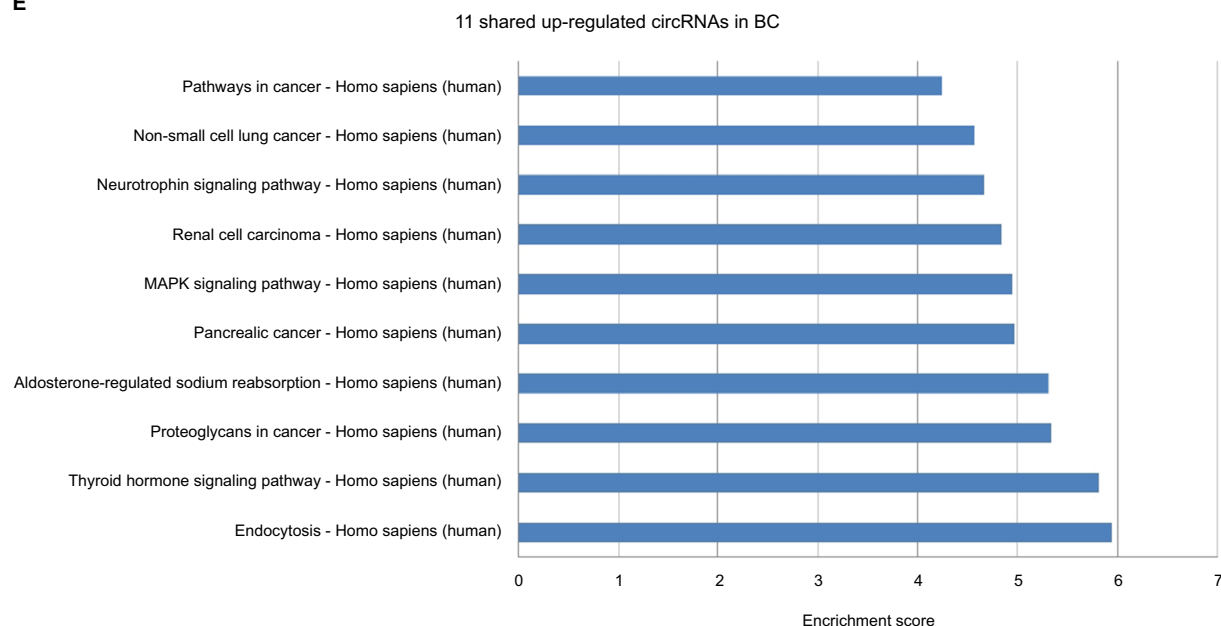

F

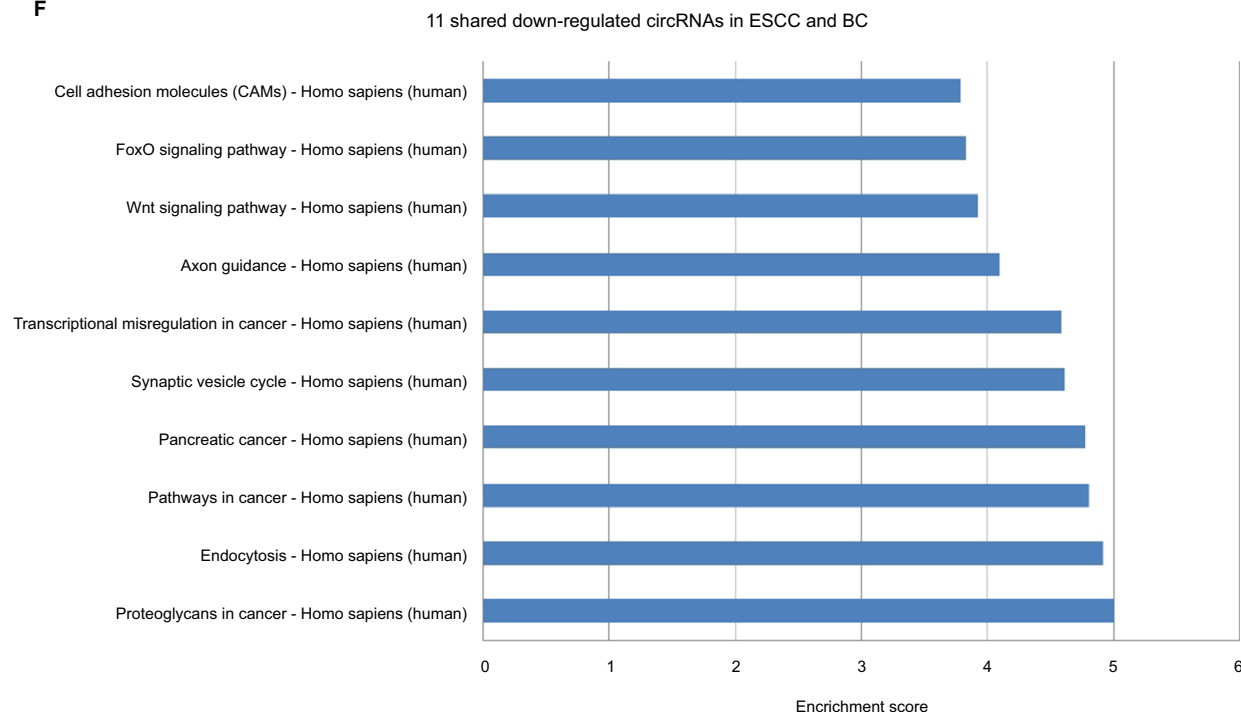## 11 shared down-regulated circRNAs in ESCC and BC

Figure S2 Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis of differentially expressed genes.
Notes: (**A**) Pathways corresponding to the top 10 upregulated circRNAs in ESCC; (**B**) Pathways corresponding to the top 10 downregulated circRNAs in ESCC; (**C**) Pathways corresponding to the top 10 upregulated circRNAs in BC; (**D**) Pathways corresponding to the top 10 downregulated circRNAs in BC; (**E**) Pathways corresponding to 11 shared upregulated circRNAs in ESCC and BC; (**F**) Pathways corresponding to 11 shared downregulated circRNAs in ESCC and BC.
Abbreviations: circRNA, circular RNA; ESCC, esophageal squamous cell cancer; BC, breast cancer.

Table S1 General information of ESCC patients for microarray test

Patient no.	Age	Ethnicity	Gender	TNM stage	Histologic differentiation	Tumor location	Histological type
1	62	Chinese Han	Male	T3N1M0	Moderate-poorly	Middle-lower	Squamous cell cancer
2	66	Chinese Han	Female	T2N0M0	Well-moderately	Middle	Squamous cell cancer
3	66	Chinese Han	Female	T3N0M0	Well-moderately	Lower	Squamous cell cancer
4	76	Chinese Han	Male	T2N0M0	Well-moderately	Middle-lower	Squamous cell cancer
5	77	Chinese Han	Female	T3N0M0	Well-moderately	Upper	Squamous cell cancer
6	69	Chinese Han	Male	T3N0M0	Well	Lower	Squamous cell cancer
7	75	Chinese Han	Female	T3N0M0	Well-moderately	Middle	Squamous cell cancer

Abbreviation: ESCC, esophageal squamous cell cancer.

Table S2 General information of BC patients for microarray test

Patient no.	Age	Ethnic	Menopausal	TNM stage	ER	PR	HER2	Histological type
1	46	Chinese Han	Pre-	T2N0M0	Positive	Positive	Negative	Invasive ductal cancer
2	62	Chinese Han	Post-	T2N0M0	Positive	Positive	Positive	Invasive ductal cancer
3	41	Chinese Han	Pre-	T2N2M0	Positive	Negative	Negative	Invasive ductal cancer
4	74	Chinese Han	Post-	T1N0M0	Negative	Negative	Positive	Invasive ductal cancer

Abbreviation: BC, breast cancer.