The effects of Stim1 and Orai1 expression levels on lymph node metastases and prognosis in patients with triple negative breast cancer

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Abstract

Objective: Calcium signaling pathways are closely related to breast cancer, including Calcium ions (Ca^{2+}) metabolic disorders associated with cell proliferation and migration of triple-negative breast cancer (TNBC). The key proteins of store-operated Ca^{2+} entry (SOCE), Stromal interaction molecule 1 (Stim1) and calcium release-activated calcium channel protein1(Orai1), play critical roles in the development of TNBC.

Method: Fifty cases of TNBC patients who had treatment in our hospital between January 2011 and January 2016 were included in the study, including no lymph node (LN) metastasis(N=20), 1-3 LNs metastasis(N=20) and ≥4 LNs metastasis(N=10). The para-cancerous tissues of TNBC patients and the plasma of healthy patients (N=20) were used as control groups for tumor tissue and plasma samples of TNBC patients, respectively. Real-time reverse transcription polymerase chain reaction (RT-qPCR) and immunohistochemical (IHC) were used to detect Stim1, Orai1 in the aforementioned tissue and plasma samples, respectively. Meantime, we used the Kaplan Meier (K-M) method to analyze the relationship between the expression levels of Stim1 and Orai1 and the prognosis of TNBC patients. Finally, the expression of SOCE and its key proteins (Stim1 and Orai1) in TNBC patients was analyzed using the TCGA database.

Main results: In TNBC patients, the expression of Stim1 and Orai1 were higher than in the control group ($P>0.05$). Besides, TNBC patients without LN metastases had higher Orai1 gene expression levels than the group with LN metastasis ($P<0.05$). The prognosis of TNBC patients is worse when the Orai1 expression is lower ($P>0.05$). Furthermore, TNBC patients with a tumor diameter≥5cm have a higher degree of axillary LN metastasis and poorer prognosis compared to patients with a diameter□5cm. On the contrary, bioinformatics analysis showed that the key protein Stim1 of SOCE was downregulated in TNBC patients and negatively correlated with the degree of lymph node metastasis, which is a protective factor in TNBC patients.

Conclusion: Orai1 is expected to be tumor markers in the field of TNBC. In addition, the Orai1 expression level and tumor diameter could be used to predict the TNBC axillary LN metastatic status and prognostic level. The relationship between Stim1 and the degree of TNBC lymph node metastasis needs further research.

1. Introduction

Calcium ions (Ca^{2+}) are important substances in human life activities, including regeneration of bone cells, protein synthesis, and contraction of various muscles. There are many mechanisms to regulate Ca^{2+} homeostasis. Interestingly, it has been revealed that store operated calcium entry (SOCE), a novel calcium pool manipulation mechanism, plays an important role in both healthy physiological functions and the development of disease[1, 2].

SOCE is mediated by the endoplasmic reticulum Ca^{2+} sensing Stim proteins, and the plasma membrane Ca^{2+} selective Orai channels. It is ubiquitously expressed in eukaryotic cells, and is the primary Ca^{2+} influx pathway in non-excitable cells. Therefore, SOCE is one of the crucial mechanisms for controlling intracellular calcium levels, which is directly associated with a variety of biological processes like gene expression and translation, cell proliferation, muscular contraction, and tumorigenesis in the body [3]. Based on the emergence and development of RNAi interference technology and high-throughput screening technology, it has been found that Stim and Orai play a significant role in the SOCE process [4, 5]. SOCE pathway refers that the interaction between Stim1 and Orai1 promotes an increase in intracellular Ca^{2+} concentration. When the photosolipase C (PLC) receptor on the plasma membrane (PM) is activated by related agonists, inositol triphosphate (IP_3) is produced, which further binds to the IP_3 receptor on the endoplasmic reticulum. And Ca^{2+} in the endoplasmic reticulum (ER) are released into the cytoplasm. The decrease of Ca^{2+} concentration in the ER is perceived by Stim1 protein, then Ca^{2+} dissociate from the N-terminal of Stim1, which leads to Stim1's conformation changes and migrates to the interface between ER and PM. Afterwards, the Stim1's C-terminal binds to Orai1, activating channel that selectively transfers Ca^{2+} from the extracellular space to the cytoplasm. This phenomenon triggers a sustained calcium release activated calcium current, increasing intracellular Ca^{2+} concentration [6].

Stim1 and Stim2, members of the evolutionarily conserved Stim family, are two single-channel membrane proteins that found on the ER [7]. Orai is a 4-fold transmembrane protein with a molecular weight of about 33 kDa that is localized in the cell membrane, and the C-terminus and N-terminus are located in the cytoplasm. Orai has three homologous proteins, of which Orai1 plays a major role in the SOCE process [8]. Stim1 and Orai1, as key calcium regulatory protein molecules, not only participate in normal physiological functions of the body, but are also closely related to the occurrence and development of tumors, with the ability to regulate apoptosis, proliferation, migration, and invasion of various cancer cells [9–13].

So far, the roles of Stim1 and Orai1 in TNBC remain unknown. Therefore, this study aims to explore the expression levels of Stim1 and Orai1 in tumor tissue and blood of TNBC patients, as well as their correlation with the degree of axillary LN metastasis. At the same time, we also analyzed the relationship between gene expression levels and the prognosis of TNBC patients, providing a new entry point for predicting the prognosis of TNBC.

2. Materials and methods

2.1 Clinical samples

Breast tumor tissues, matched para-cancerous tissues and plasma were obtained from 50 cases of TNBC patients admitted between January 2011 and January 2016 were collected from the bio-sample bank of Zhejiang Cancer Hospital. All patients were diagnosed with TNBC. They got no chemotherapy or radiotherapy prior to surgery, and all patients underwent a radical mastectomy for breast cancer while

hospitalized. Twenty of the 50 cases samples had no LN metastasis, 20 cases had 1–3 LN metastasis, and 10 cases had ≥ 4 LN metastasis. Using the plasma of 20 cases healthy individuals as the control group for TNBC patients' plasma.

2.2 Clinical data

Clinically relevant data of the patients were collected and collated, including patients' age, menstrual status, age at menarche, reproductive history, family history of malignant tumor, family history of breast cancer, tumor location, maximum diameter of tumor, numbers of LN metastases, Ki-67 expression level, surgical methods, and whether chemotherapy after surgery.

By October 2022, we had conducted outpatient or telephone follow-up of all patients, including the survival of patients, postoperative tumor recurrence and metastasis. Overall survival (OS) refers to the number of months between radical breast cancer surgery and death. Recurrence free survival (RFS) measures the number of months between radical surgery and the first breast cancer recurrence, metastasis, or death.

2.3 Real-time reverse transcription polymerase chain reaction (RT-qPCR)

RNA was extracted from tissue and plasma samples according to the standard procedure of the Animal Total RNA Rapid Extraction Kit (Shanghai Jieri # GK3016), and the concentration and purity of the extracted RNA were determined in the Ultra-Micro Nucleic Acid Analyzer (Nano-400A). The total RNA (1 μg) was reverse transcribed with a reverse transcription kit (Nanjing Novozymes # R212-02) to synthesize cDNA. Using cDNA as a template, real-time PCR was performed by a qPCR kit (Nanjing Novozan # Q711-02) and a real-time fluorescence quantitative PCR instrument (Accurate96-x4). Finally, based on the obtained Ct values, we calculate the relative expression level of genes using the $2^{-\triangle\triangle Ct}$ method. The relative expression of the genes is listed in Supplementary Table 1.

2.4 Immunohistochemical (IHC)

IHC determination of the samples was carried out using a two-step kit from Beijing Zhongshan Biotechnology Co. Stim1 and Orai1 antibodies were purchased from Abcam, and the second antibody was from Zhongshan Biotechnology, Beijing. The cytoplasm is yellow or yellow brown, and the nucleus is blue, indicating IHC (+). Positive intensity and the number of positive cells were scored separately. No positive cells were scored as 0, positive cells ≤ 25% were scored as 1, 26%-50% were scored as 2, 51%-75% were scored as 3, and > 75% were scored as 4. The intensity of coloring was scored as 0 for colorless, 1 for pale yellow, 2 for tan, and 3 for brown, and finally the product of the two was used as the expression of Stim1 or Orai1.

2.5 Bioinformatics analysis

We downloaded gene expression data and clinical follow-up information of the TCGA breast cancer (BRCA) dataset from the USCS Xena database, especially the IHC receptor assay results of breast cancer

patients and the number of positive lymph nodes in patients ("number of lymph nodes positive by he"). And we download (GOBP STORE OPERATED CALCIUM ENTRY and GOBP REGULATION OF STORE OPERATED CALCIUM ENTRY) group from the MSigDB (http://software.broadinstitute.org/gsea/MSigDB) database. The "gsva" algorithm in the R package "GSVA" (version: 1.42.0) was applied to quantitatively score the samples, and the Wilcoxon rank sum test was applied to compare the differences between the samples of different subtypes of patients. Analysis of Variance (ANOVA) was applied to compare the differential expression between normal samples and patients with the four breast cancer subtypes, and the p-values were recorded. The Pearson correlation coefficient (PCC) between Stim1 gene expression and the number of positive lymph nodes was calculated, and a correlation test (cor. test) was performed. Based on two GSVA scores, the samples were divided into high and low expression groups. A single factor Cox proportional analysis model was constructed using R-package "survival" (Version: 3.3-1) and a log rank test was performed to record the disease specific survival (DSS) Hazard Ratio (HR) [95% CI] and p-value of the corresponding genes.

2.6 Data analysis

One-way ANOVA, Student's t-test, chi-square test, Spearman rank correlation, COX regression and two-tailed distributions were used to calculate the statistical significance. Analyze the significance of K-M survival curve using the log Rank method. These analyses were done with GraphPad Prism 8.0 and SPSS Statistics 25. Results were considered statistically significant when p-values < 0.05 between groups. Using R packages such as RMS, surveyors, ggplot2, and ggsci, the linear or nonlinear relationship between Stim1 and Orai1 expression levels and survival and recurrence risk HR was tested after adjusting for menstrual status and family history of breast cancer.

3. Results

3.1 Stim1 and Orai1 are highly expressed in TNBC tumor tissues and plasma

We detected the expression levels of the Stim1 and Orai1 in 50 tumor tissue samples and para-cancerous tissues by RT-qPCR. The results revealed that the expression levels of Stim1 and Orai1 in breast tumor tissue without lymph node metastasis were higher than those in para-cancerous breast tissues ($P > 0.05$). In 20 paired samples with 1–3 lymph node metastases, the same result has been achieved, but only Stim1 showed statistical differences ($P < 0.05$). In 10 paired samples with ≥ 4 lymph node metastases, the expression levels of Stim1 and Orai1 in breast tumor tissue were still higher than those in normal tissue ($P > 0.05$). Although there was no statistical difference in many groups, the Stim1 and Orai1 overall expression trend were that tumor tissues were higher than para-cancerous tissues. (Fig. 1)

The expression levels of Stim1 and Orai1 in the plasma of 20 cases of TNBC patients without lymph node metastases were found to be considerably higher than control group ($P < 0.05$). The expression

levels of Stim1 and Orai1 genes in the plasma of TNBC patients with 1–3 lymph node metastasis were higher than those of healthy women, but only STIM1 was statistically significant ($P < 0.05$). Stim1 and Orai1 expression levels in plasma with ≥ 4 lymph node metastases also exhibited a comparable trend. Overall, the expression levels of the Stim1 and Orai1 genes in the plasma of TNBC patients with different degrees of lymph node metastasis were higher than those in the control group. (Fig. 2)

3.2 Orai1 overexpression in TNBC without lymph node metastasis

Further analysis of the differences in Stim1 and Orai1 gene expression levels between the three aforementioned groups revealed that there was a statistically significant difference in Orai1 gene expression levels between tumor tissue and plasma samples without lymph node metastasis and 1–3 lymph node metastasis ($P < 0.05$), but not between the other groups. Additionally, it was not discovered that there was a linear relationship between the two genes. (Fig. 3)

We further combined 1–3 LN metastases with ≥ 4 LN metastases to form a group with LN metastasis, and compared the expression levels of Stim1 and Orai1 with the group without LN metastasis. The results of tumor tissue testing showed that the expression level of Stim1 was lower in the group without LN metastasis than in the group with LN metastasis ($P > 0.05$). On the contrary, the expression level of Orai1 was higher in the group without LN metastasis than in the group with LN metastasis ($P < 0.05$). Besides, the expression of Stim1 and Orai1 genes in TNBC plasma of the group without LN metastasis was higher than that of the group with LN metastasis, and only Orai1 expression showed statistical differences ($P < 0.05$). (Fig. 4)

Furthermore, we performed IHC detection of Stim1 and Orai1 proteins in tumor tissue from three groups with three degrees of lymph node metastasis (Figs. 5, 6), which revealed that the expression of Stim1 and Orai1 proteins decreased with increasing lymph node metastasis ($P > 0.05$). (Fig. 7)

3.3. The impact of Stim1 and Orai1 on prognosis

We divided the expression levels of Stim1 and Orai1 into high expression and low expression groups (The median is the cutoff point) for survival analysis. The results showed that the OS and RFS of the Stim1 high expression group were lower than those of the low expression group in the tumor tissues, and the Orai1 high expression group had a longer OS and RFS than the low expression group ($P > 0.05$). (Fig. 8)

When Stim1 expression was lower than 2.5, according to RCS analysis of Stim1 and Orai1 IHC data, the risk of death was virtually unaffected. When Stim1 expression was greater than 2.5, the risk of mortality increased along with it (Fig. 9a). The risk of death score was essentially unaffected when Orai1's value fell below 0.5. Additionally, when Orai1 was greater than 0.5, the possibility of death first rose before trending downward as Orai1 increased (Fig. 9b). The recurrence risk trend of Stim1 and Orai1 is consistent with the survival risk. (Fig. 9c, d).

3.4 Clinical data analysis:

3.4.1 Basic characteristics

50 women with breast cancer diagnosed as TNBC were included in the statistical analysis. Among of them, 19 cases (38%) were under 50 years old, 32 cases (64%) were menopausal, 10% had menarche prior to the age of 15, and 15 cases (30%) had a family history of malignant tumors. The majority of patients (62%) had tumors in the outer upper quadrant, 4 (8%) patients had a family history of breast cancer, 22 (44%) patients had tumors in the left breast, 48 (96%) patients with TNBC underwent total mastectomy, and 44 (88%) patients obtained postoperative chemotherapy. (Table 1)

Table 1
Basic characteristics of TNBC

Characteristics	No. of cases (%)
Patients(N)	50
Age	
< 50	19(38.0)
≥ 50	31(62.0)
Menstruation	
Yes	18(36.0)
No	32(64.0)
Age of menarche	
< 15	10(20.0)
≥ 15	40(80.0)
Family history of breast cancer	
Yes	4(8.0)
No	46(92.0)
Family history of malignant tumors	
Yes	15(30.0)
No	35(70.0)
Tumor site	
Left	22(44.0)
Right	28(56.0)
Tumor quadrant	
Outer upper quadrant	31(62.0)
Outer lower quadrant	4(8.0)
Inner upper quadrant	11(22.0)
Inner lower quadrant	4(8.0)
operation	
Breast conserving therapy	2(4.0)
Mastectomy	48(96.0)

Characteristics	No. of cases (%)
Chemotherapy	
Yes	44(88.0)
No	6(12.0)

3.4.2 The influencing factors of lymph node metastasis

Age, menstrual status, age at menarche, family history of malignant tumors (including family history of breast cancer), tumor location, Ki-67 expression, and tissue IHC results among the three groups did not statistically differ from one another in the univariate analysis of the variables mentioned ($P > 0.05$). In different lymph node metastasis groups, the outcomes of the histological PCR showed no statistically significant difference between the Stim1 high expression group and the Stim1 low expression group ($P > 0.05$). However, the expression of Orai1 was significantly different in the three different degrees of lymph node metastasis status ($P < 0.05$) and was highly expressed in the no lymph node metastasis status. The outcomes of the plasma test confirmed the same findings. The maximum diameter of the tumor was statistically different between the groups with different degrees of lymph node metastasis ($P < 0.05$). After multiple comparisons, it was found that the group with no lymph node metastasis was significantly different from the group with ≥ 4 lymph nodes metastasis (Table 2).

Perform correlation analysis on three data with statistical differences in univariate analysis. The results showed a negative correlation between the expression level of Orai1 in tissues and plasma and the degree of lymph node metastasis. The maximal tumor diameter is positively connected with the degree of lymph node metastasis ($P = 0.003$). The findings suggest that Orai1 and tumor diameter have differing effects on lymph node metastasis in TNBC patients. The lower the level of Orai1 expression, the higher the degree of lymph node metastases in TNBC patients. The degree of lymph node metastasis in TNBC patients with a tumor diameter ≥ 5cm is higher than that in TNBC patients with a tumor diameter < 5cm. (Table 3)

We further conducted univariate COX regression analysis on the three variables with statistical differences, and the results showed that the survival time of the high expression group of Orai1 in tissues was higher than that of the low expression group ($P < 0.05$, HR = 4.295, 95% CI 1.175–15.695). In addition, the patients with larger tumor diameter showed shorter survival time ($P < 0.05$, HR = 3.77, 95% CI 1.156–12.294), while the expression level of Orai1 in plasma had no statistically significant impact on patient survival. The results of multivariate analysis showed that the expression level of Orai1 in tumor tissue and plasma, as well as tumor diameter, had no statistically significant impact on the prognosis of patients($P > 0.05$). (Table 4)

Table 2
Univariate analysis

	Lymph node metastasis status			
	0	1–3	≥ 4	P value
Age				
< 50	9	5	5	0.29
≥ 50	11	15	5	
Menstruation				
Yes	9	4	5	0.15
No	11	16	5	
Age of menarche				
< 15	5	2	3	0.38
≥ 15	15	18	7	
Family history of breast cancer				
Yes	1	1	2	0.32
No	19	19	18	
Family history of malignant tumors				
Yes	4	7	4	0.44
No	16	13	6	
Tumor site				
Left	9	9	4	0.96
Right	11	11	6	
Tumor quadrant				
Outer upper quadrant	10	13	8	0.45
Outer lower quadrant	1	3	0	
Inner upper quadrant	7	3	1	
Inner lower quadrant	2	1	1	
Stim1(tissue PCR)				
High	11	10	4	0.74

	Lymph node metastasis status			
	0	1–3	≥ 4	P value
Age				
Low	9	10	6	
Orai1(tissue PCR)				
High	15	5	5	0.007
Low	5	15	5	
Stim1(plasma PCR)				
High	12	8	5	0.45
Low	8	12	5	
Orai1(plasma PCR)				
High	16	1	8	0.000
Low	4	19	2	
Stim1(IHC)				
High	12	10	3	0.41
Low	8	10	6	
Orai1(IHC)				
High	12	8	5	0.43
Low	8	12	4	
Tumor diameter				
d < 5cm	20	18	6	0.01
d ≥ 5cm	0	2	4	
Ki-67				
High	11	10	3	0.42
Low	9	10	7	

Table 3
Correlation analysis

Variables	Lymph node metastasis status	
	Spearman correlation	P value
Orai1(tissue PCR)	-0.298	0.04
Orai1(plasma PCR)	-0.224	0.12
Tumor diameter	0.413	0.003

Table 4
COX Survival Analysis

	Univariate analysis			Multivariate analysis		
	P	Hazard Ratio	95% confidence interval	P	Hazard Ratio	95% confidence interval
Orai1(tissue PCR)	0.03	4.295	1.175–15.695	0.08	3.366	0.876–12.927
Orai1(plasma PCR)	0.35	1.709	0.559–5.226	0.46	1.538	0.489–4.845
Tumor diameter	0.03	3.77	1.156–12.294	0.08	3.036	0.889–10.369

4. Bioinformatics analysis

We obtained and downloaded The Cancer Genome Atlas (TCGA) BRCA dataset from the UCSC Xena database, which included patient gene expression data and clinical prognostic information, as well as results of human epidermal growth factor receptor 2 (Her-2/neu), progesterone receptor (PR), and estrogen receptor (ER) identification in IHC for each patient. Two related gene sets, "GOBP STORE OPERATED CALCIUM ENTRY" and "GOBP REGULATION OF STORE OPERATED CALCIUM ENTRY" were collected and downloaded from the MSigDB database to analyze the differences of SOCE among patients with different subtypes of breast cancer. Using the "GSVA" R package, the samples in the TCGA BRCA dataset were quantitatively assessed and compared using the Wilcoxon rank sum test for differences. The results showed that the GSVA score in the normal population was higher than that in the patient sample, and the score in TNBC subtype patients was the lowest, compared to the normal population, with p-values of 3.5e-04 and 3.4e-12, respectively, (Fig. 10). This demonstrates a considerable inhibition of SOCE function in TNBC subtype patients.

Further disease specific survival (DSS) analysis was performed on the two GSVA scores in the aforementioned patients by combining the prognostic follow-up information recorded in the TCGA BRCA dataset sample. Firstly, based on the GSVA score of "GOBP STORE ORATED CALCIUM ENTRY", patients

were divided into high and low risk groups with a threshold of -0.2677. The HR [95% CI] of the final constructed univariate Cox proportional risk model was 0.4164 [0.2519–0.6884], and the p-value of the log rank test was 0.0062 (Fig. 11). Subsequently, according to the GSVA score of "GOBP REGULATION OF STORE OPERATED CALCIUM ENTRY", patients were divided into high and low risk groups with a threshold of -0.1725. The HR [95% CI] of the final constructed single factor Cox proportional risk model was 0.5230 [0.3405 0.8034], and the p-value of the log rank test was 0.0062 (Fig. 11). It indicates that SOCE is a significant protective factor in breast cancer patients, whose abnormal down-regulation in TNBC patients potentially indicates the poor prognosis.

Based on the aforementioned analysis, we went on to screen the key genes of the SOCE that were significantly inhibited in TNBC patients, count the mean expression in both normal individuals and TNBC patients, and use ANOVA to analyze the differential expression between individuals without breast cancer and those with four different subtypes. Nine of these genes were shown to be considerably downregulated in the patients with TNBC ($P < 0.01$). The Stim protein family (Stim1 and Stim2) were down regulated, with FC values of 0.9600 and 0.8765 for, respectively. However, Orai family's expression was elevated. (Table 5)

Table 5
SOCE related genes downregulated in TNBC patients

gene	mean expression of Normal	mean expression of TNBC	FC	P value
EFHB	16.0978	10.0469	0.6241	1.2261E-09
CASQ1	17.1734	10.8303	0.6306	1.1912E-14
JPH4	22.1840	15.4845	0.6980	2.8191E-29
STC2	89.2352	69.8242	0.7825	7.1291E-44
SPG11	88.1463	76.6980	0.8701	1.2090E-71
STIM2	66.1004	57.9391	0.8765	1.3448E-28
ORAI3	58.6395	51.6141	0.8802	2.9069E-43
SPINK1	8.2342	7.3598	0.8938	6.0143E-04
STIM1	87.8981	84.3837	0.9600	8.1336E-18

119 cases of TNBC patients were also evaluated in the TCGA BRCA dataset, both for Stim1 and Stim2 expression as well as predictive follow-up data. A disease-specific survival analysis of the expression of two genes in the aforementioned patients was then performed. Firstly, patients were divided into groups with high and low levels of Stim1 expression using a threshold of 81.7211. The final univariate Cox proportional risk model had an HR [95% CI] of 0.1934 [0.0530–0.7052] and a p-value of 0.0056 for the log rank test (Fig. 12). Patients were then split into groups with high and low levels of Stim2 expression using a cutoff of 58.1913. The final univariate Cox proportional risk model had an HR [95% CI] of 0.3594

[0.0985–1.3110], and the log rank test had a p-value of 0.1100 (Fig. 12). This suggests that the Stim protein family, whose increased expression improves patient prognosis, is a protective factor in breast cancer patients.

Additionally, research has demonstrated an association between improper calcium homeostasis and lymph node metastases[14]. Utilizing the "number" in clinical data from patient records from the TCGA BRCA dataset of "number of lymph nodes positive by he". Our calculations of the Pearson correlation coefficient (PCC) between the expression of the Stim1 gene and the number of positive lymph nodes revealed a significant negative correlation between the two (Fig. 13), with a PCC of -0.1208 and a correlation test p-value of 0.0029. It was observed that the majority of the samples were 0, which can have an impact on the correlation study' findings. The threshold of 3 positive lymph nodes in the patient was used for high and low classification. Subsequently, the Wilcoxon rank sum test was used for difference comparison. The results showed that there was significantly lower Stim1 gene expression in patients with 3 or more positive lymph nodes, with a p-value of 0.0041 (Fig. 13). We also performed a correlation study for Orai1, but the results showed no relationship between the number of positive lymph nodes and the PCC was − 0.0259 and the p-value of the test was 0.5248.

On this basis, we would like to further analyze the correlation between Stim1 and TNBC lymph node metastasis. However, no expression of Stim1 was detected of GSE184720 and GSE180286, which were collected and downloaded from the GEO database. Moreover, in the previous analysis of the GSE61723 dataset, Stim1 and Orai1 and Orai3 did not show significant differential expression in TNBC samples with lymph node metastasis. (Table 6)

Table 6
Expression of SOCE related genes in the GSE61723 TNBC dataset

Gene	Without LNs metastasis	With LNs metastasis	Fold Change	P value
STIM1	6.580304048	6.363295157	0.967021449	0.548113815
STIM2	6.03145172	6.891791202	1.14264219	0.402184182
ORAI2	4.70182714	4.750097886	1.01026638	0.84311778
HOMER1	12.5678856	11.42071318	0.908721924	0.100165758
ORAI1	6.396352726	8.094920984	1.265552625	0.095562655
JPH4	7.720923796	8.081015309	1.046638397	0.250517403
ORAI3	4.893383437	4.828640628	0.986769316	0.929917878
HOMER2	6.811077352	5.613683956	0.824199119	0.440544356
CD84	4.916541007	4.949063655	1.006614945	0.440544356

TNBC: Triple negative breast cancer; LNs: lymph nodes.

Through the aforementioned investigation, we discovered that TNBC patients' SOCE processes were significantly hindered as compared to those without TNBC. Stim1 was a key protective factor that was downregulated in TNBC patients. Stim1 and Orai1 are not significantly linked to lymph node metastases in TNBC patients, according to several research. The summary of the results is detailed in Table 7.

Table 7
Summary of Stim1 and Orai1 expression in breast cancer

Group	Factors	Sample	STIM1	ORAI1	From
TNBC tumor tissue (compared to self-cancerous tissue)	Gene	Tissue	High	High	This project
TNBC plasma (compared to healthy plasma)	Gene	Plasma	High	High	This project
TNBC with no LNs metastasis compared to with LNs metastasis	Degree of lymph node metastasis	Tissue	The higher the expression level in tissues with LNs metastasis	The lower the expression level in tissues with LNs metastasis	This project
TNBC with no LNs metastasis compared to with LNs metastasis	Degree of lymph node metastasis	Plasma	The lower the expression level in tissues with LNs metastasis	The lower the expression level in tissues with LNs metastasis	This project
Gene (Stim1 and Orai1) high expression group and low expression group	DFS, OS	Tissue	The higher the expression level, the worse the prognosis	The higher the expression level, the better the prognosis	This project
Healthy people and four breast cancer subtypes	Gene	TCGA	Downregulated in TNBC	Upregulated in TNBC	This project
Stim1 high expression group and low expression group in TNBC	DFS, OS	TCGA	The higher the expression, the better the prognosis of patients	-	This project
≥ 3 LNs compare to<3 LNs	Degree of lymph node metastasis	TCGA	Patients with ≥ 3 LNs metastases have lower Stim1 gene expression	No correlation	This project
TNBC	Degree of lymph node metastasis	GSE184720 and GSE180286	Not detected	---	This project
TNBC and other types of	Gene	Tumor tissue	High expression in TNBC	---	Yang et al.,

Group	Factors	Sample	STIM1	ORAI1	From
breast cancer					2017
Breast cancer	OS, DFS	TCGA	High expression patients have a shorter survival time	---	Huang et al., 2020
Breast cancer	OS, DFS	TCGA and GTEx	The higher the expression, the better the prognosis	---	Zhang, et al., 2023
TNBC and other types of breast cancer	Gene	TCGA	---	High expression in TNBC	Azimi et al., 2019

TNBC: Triple negative breast cancer; LNs: lymph nodes; OS: Overall Survival, DFS: Disease-free survival.

5. Discussion

SOCE is one of the important mechanisms for regulating calcium concentration in cells. Stim1, as a key calcium regulatory protein molecule, is not only involved in normal physiological functions of the body, but also closely related to the occurrence and development of tumors. It has the ability to regulate apoptosis, proliferation, migration, and invasion of various cancer cells[15]. There are several relevant literature reports that Stim1 is highly expressed in TNBC cell[3] [9]. Stim1 expression in breast cancer tumor tissue was significantly higher than that in para-cancerous tissue, and its expression in TNBC tumor tissue was significantly higher than that in other subtypes of breast cancer [16]. The findings of this study also revealed that Stim1 was highly expressed in the tumor tissues of TNBC patients (Compared to adjacent tissues). We have also added IHC of TNBC tumor tissue and PCR of Stim1 in patient plasma. In addition, we also conducted multi-dimensional comparisons and validation by combining bioinformatics analysis.

Breast cancer cell migration can be aided by Stim1[17–19]. The crucial function of Stim1 in the migration of breast cancer cells was initially established by Yang et al. They discovered that Stim1 can encourage TNBC cells to migrate, and they proceeded on to employ RNA interference (RNAi) technology to knockdown Stim1 in MDA-MB-231 cells. They discovered that this greatly decreased the cells' capacity to migrate[10]. However, several pertinent research have demonstrated that Stim1 can prevent breast cancer cells from migrating and invading. For instance, Zhang et al. discovered that the later the tumor stage in invasive breast cancer, the lower the expression of Stim1 was[15]. Furthermore, H et al. discovered that deleting Stim1 entirely in metastatic TNBC cells caused them to migrate more quickly and to be more invasive[20].

The biological role of Stim1 in breast cancer, especially in TNBC, is still controversial. The results of this experiment show that the expression level of the Stim1 gene in TNBC tumor tissue is higher in the group

with lymph node metastasis than in the group without lymph node metastasis (P > 0.05), while the IHC results show that the expression level of the Stim1 protein is lower in the group with lymph node metastasis (P > 0.05). The expression level of Stim1 in the patient's plasma is similar to the IHC results. The reason for the inconsistency between PCR results and IHC results may be 1. The sample size of this study is too small; 2. The degree of protein degradation in tissue wax blocks is faster than in frozen samples; 3. Sample storage time too long. In the later stage, we need to expand the sample size and collect recent frozen samples for further research.

Huang et al. analyzed the prognosis of breast cancer patients in the Stim1 high expression group (N = 208) and low expression group (N = 317) in the TCGA database, and the results showed that the Stim1 high expression group had a lower survival time than the low expression group [21]. Yang et al. discovered the high expression of Stim1 was linked to poor OS and DFS in breast cancer patients [16]. Patients with high Stim1 expression, on the other hand, appear to have a better prognosis according to certain research. For example, Zhang et al. employed the K-M approach to investigate the association between Stim1 expression levels and patient prognosis in BC patients after collecting Stim1 data from the TCGA and GTEx databases. The findings indicated that prolonged OS and DFS in BC patients were associated with increased Stim1 expression levels [15]. Although Stim1 is linked to a poor outcome in TNBC patients according to our data, the difference is not statistically significant.

The SOCE was revealed to be impeded in TNBC using bioinformatics analysis, and the SOCE process may operate as a preventative measure for breast cancer. The study of SOCE key genes revealed that Stim protein acts as a preventative measure against breast cancer, the higher it’s expression level, the better the prognosis of patients. Stim1 is inversely linked with the extent of lymph node metastases in breast cancer. The results of the experiment are in contrast to those of the aforementioned bioinformatics analysis. The specific reason cannot be explained at present, we will expand the sample size to further explore.

Orai has a crucial role in the onset and progression of cancer [22]. Three similar proteins belonging to the Orai family are found in mammals: Orai1, Orai2, and Orai3 [23, 24]. Elevated Orai1 is a sign of TNBC [25, 26]. Orai1 was more expressed in TNBC tumor tissue and plasma than in the control group, according to the experiment's results, which are consistent with earlier literature studies and bioinformatics analysis results, suggesting that Orai1 may serve as a tumor marker.

The association between Orai1 and the presence of axillary lymph node metastases in TNBC patients has not yet been the subject of any study publications. The findings of this study demonstrated that, despite the lack of a direct relationship between the degree of lymph node metastasis in the three groups and the level of Orai1 expression, when we compared the groups with and without lymph node metastasis, we discovered that the expression level of Orai1 in tumor tissue or plasma was lower in the group with lymph node metastasis. Additionally, patients with high Orai1 expression had better prognoses than those with low expression, suggesting that Orai1 has the ability to block tumor migration and influence the prognosis of TNBC. However, Liu X et al, and other researchers have shown that

hypoxia can increase the expression of the Orai1 gene by activating the Notch pathway, boosting the invasion and migration of TNBC cells and potentially treating TNBC through the target of Orai1[27]. Additionally, SC et al. demonstrated that Orai1 can be activated by adenyl cyclase type 8 (AC8), which encourages the SOCE process in TNBC cells, raises intracellular calcium ion concentration, and encourages TNBC cell migration [25]. The reasons for the differences in the above research results may be: 1. The pathway for increasing intracellular Ca^{2+} concentration is not only through SOCE, but also through many other pathways, such as calcium pumps, Na^{+}- Ca^{2+}channels, calcium leakage channels, and so on[28].; 2. Stim1 and Orai1 are just two important proteins in the process of SOCE, and multiple factors such as activators, phospholipase C (PLC) receptors, and nuclear factor of activated T-cells 1 (NFAT) co-regulate the pathway [6, 29]. In order to confirm the experimental outcomes of histology, we will also carry out cell experiment in the future.

Han et al. analyzed the relationship between Her-2 expression and tumor diameter in 217 cases of breast cancer patients and found that if the tumor diameter is larger, the Her-2 expression is the lower in tumor [30]. TNBC is a subtype of breast cancer with negative expression of Her-2, PR, and ER. It grows faster, has a higher malignancy rate and a larger tumor diameter compared with other types of breast cancer[31, 32]. According to relevant literature reports, in TNBC patients, the larger the tumor diameter, the higher the degree of lymph node metastasis, and the poorer the prognosis [33]. The results of this study are consistent with previous literature reports that tumor diameter is positively correlated with lymph node metastasis status in TNBC patients and negatively correlated with prognosis level.

Overall, our study's findings show that the expression of Orai1 in patient tissues and plasma may one day function as tumor marker in TNBC research. However, more research is required to determine whether Orai1 expression patterns differ in other cancer cells. As for the correlation between Stim1 expression level and TNBC and the degree of TNBC lymph node metastasis, further research is needed. The degree of Orai1 expression and the tumor's maximum diameter both have an impact on the metastasis of the axillary lymph nodes. Better prognosis is achieved in TNBC patients with higher Orai1 expression levels, smaller tumor diameter, and lower lymph node metastatic status. In the future, we can also develop a prediction model based on the Orai1 expression level and tumor diameter to more precisely forecast the prognosis of TNBC and the status of axillary lymph node metastases. Additionally, we intend to investigate the mechanism in depth and better examine how Orai1 work.

6. Conclusion

In conclusion, our research's findings show that Stim1 and Orai1 are both significantly expressed in TNBC tumor tissue or plasma (Compared to adjacent tissues), Orai1 has a lower expression level in TNBC samples with lymph node metastasis ($P < 0.05$). Additionally, there is a negative correlation between prognosis level and tumor diameter and a positive correlation between the condition of TNBC axillary lymph nodes. These studies suggest that Orai1 may develop as an oncological marker for identifying TNBC. Moreover, analyzing the expression level of Orai1 and the maximum diameter of the tumor can effectively predict whether axillary lymph node metastasis and prognosis level in TNBC

patients. For Stim1, bioinformatics analysis showed that it plays a protective role in the occurrence and development of TNBC, while histological PCR and K-M survival analysis showed the opposite results. So in order to understand its specific role in TNBC, we still need further research.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Zhejiang Cancer Hospital (Ethics Number: IRB-2020-313). Additional informed consent was obtained from all patients for which identifying information is included in this article.

Consent for publication

Not applicable

Data Availability and materials

The data that support the findings of this study are available on request from the corresponding author, Xi-ping Zhang upon reasonable request.

Competing interests:

Qiu-hui Yang, Hong-jian Yang, Ye-qin Fu, Wen-ju Mo, Chen Wang, Jie-fei Mao, Xi-ping Zhang declare that they have no conflict of interest.

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Author contributions

Qiu-hui Yang: Writing–original draft, Conceptualization, Formal Analysis.

Hong-jian Yang: Conceptualization, Supervision, Writing – review & editing.

Ye-qin Fu: Writing – review.

Wen-ju Mo: Software, Visualization.

Chen Wang: Conceptualization, Visualization.

Jie-fei Mao: Writing – review & editing.

Xi-ping Zhang: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Figures

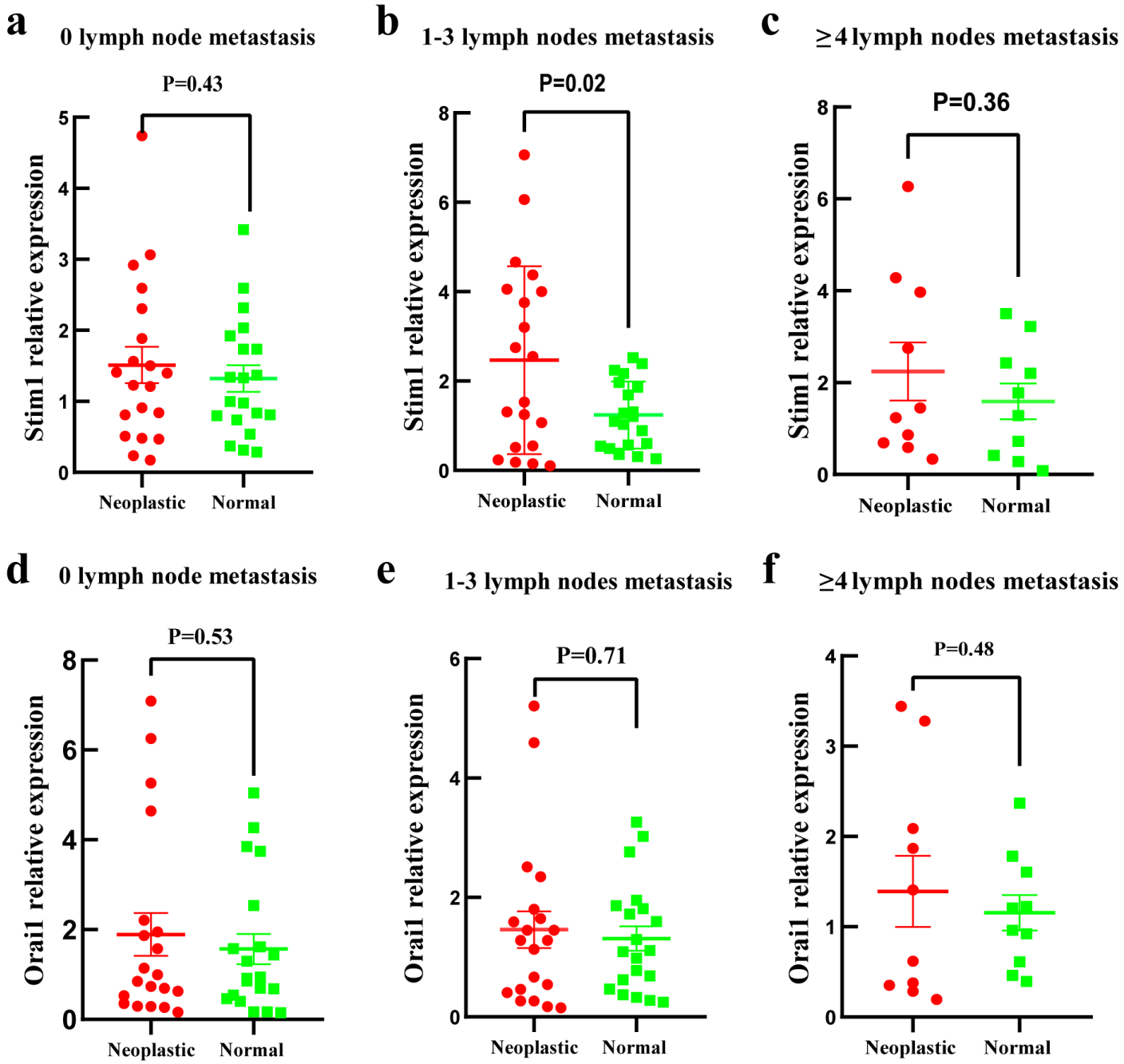

Figure 1

RT-qPCR results of breast tissue samples. a. Comparison of Stim1 expression levels between TNBC tumor tissue without lymph node metastasis and para-cancerous tissues. b. Comparison of Stim1 expression levels between TNBC tumor tissue and para-cancerous tissues with 1-3 lymph node

metastases. c. Comparison of Stim1 expression levels between TNBC tumor tissues and para-cancerous tissues with ≥4 lymph node metastases. d. Comparison of Orai1 expression levels between TNBC tumor tissue without lymph node metastasis and para-cancerous tissues. e. Comparison of Orai1 expression levels between TNBC tumor tissue and para-cancerous tissues with 1-3 lymph node metastases. f. Comparison of Stim1 expression levels between TNBC tumor tissue and para-cancerous tissues with ≥4 lymph node metastases.

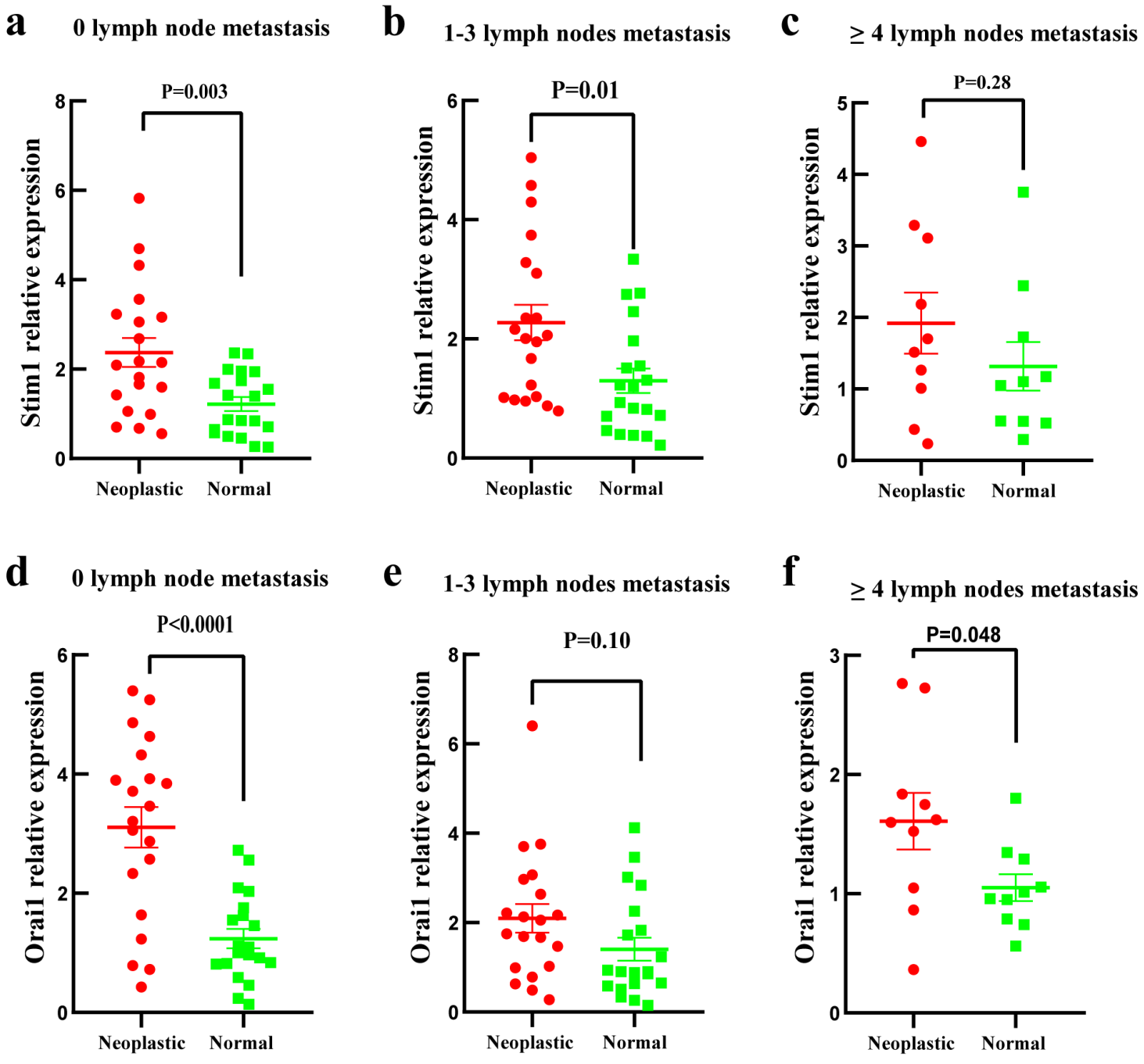

Figure 2

RT-qPCR results of plasma samples. a. Comparison of Stim1 expression levels in plasma of TNBC patients without lymph node metastasis and healthy plasma. b. Comparison of plasma Stim1 expression

levels between TNBC patients with 1-3 lymph node metastasis and healthy women. c. Comparison of Stim1 expression levels in the plasma of TNBC patients with ≥4 lymph node metastases and healthy plasma. d. Comparison of Orai1 expression levels in plasma of TNBC patients without lymph node metastasis and healthy plasma. e. Comparison of Orai1 expression levels in the plasma of TNBC patients with 1-3 lymph node metastases and healthy plasma. f. Comparison of Stim1 expression levels in plasma of TNBC patients with ≥4 lymph node metastases and healthy plasma.

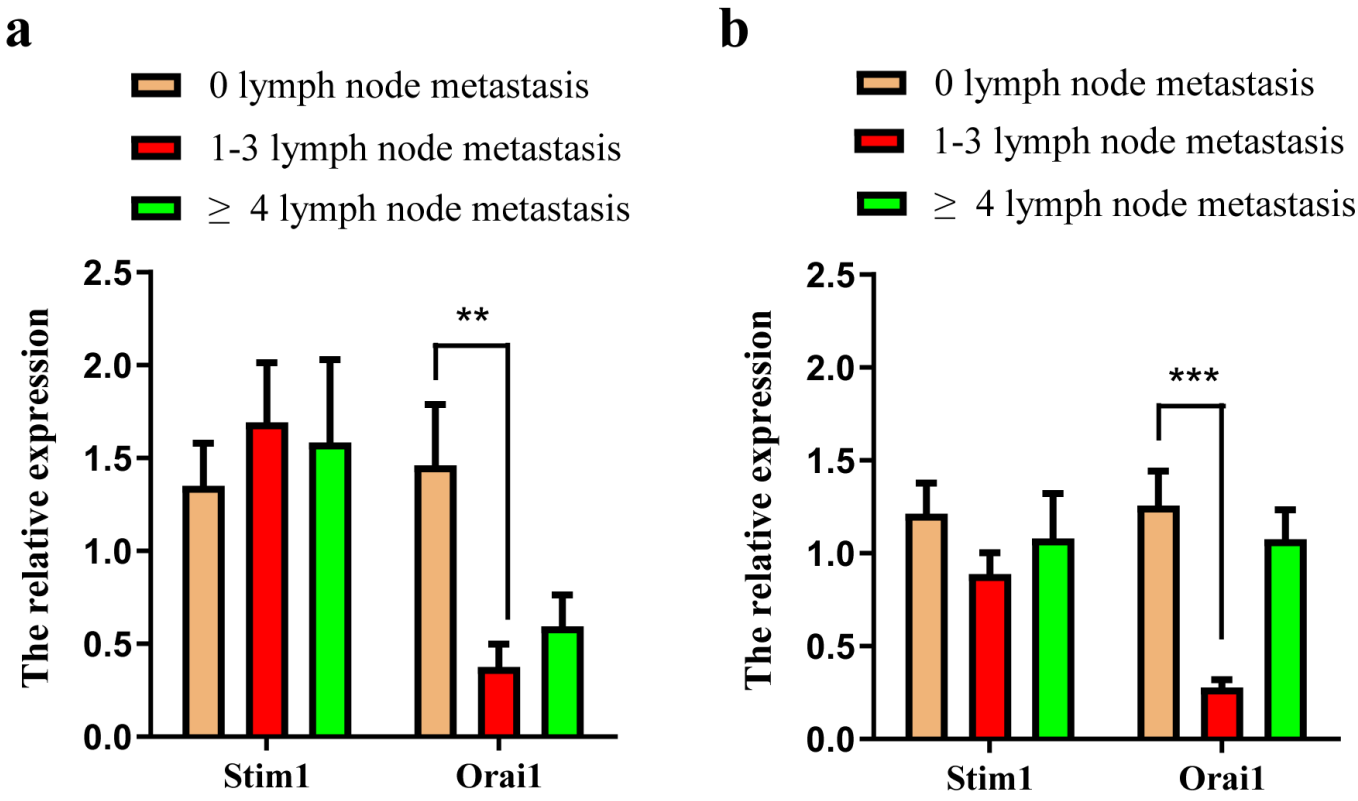

Figure 3

Comparison of Stim1 and Orai1 gene expression levels in tissues and plasma among groups. a. Comparison of Stim1 and Orai1 expression levels in tumor tissue. b. Comparison of Stim1 and Orai1 expression levels in plasma.

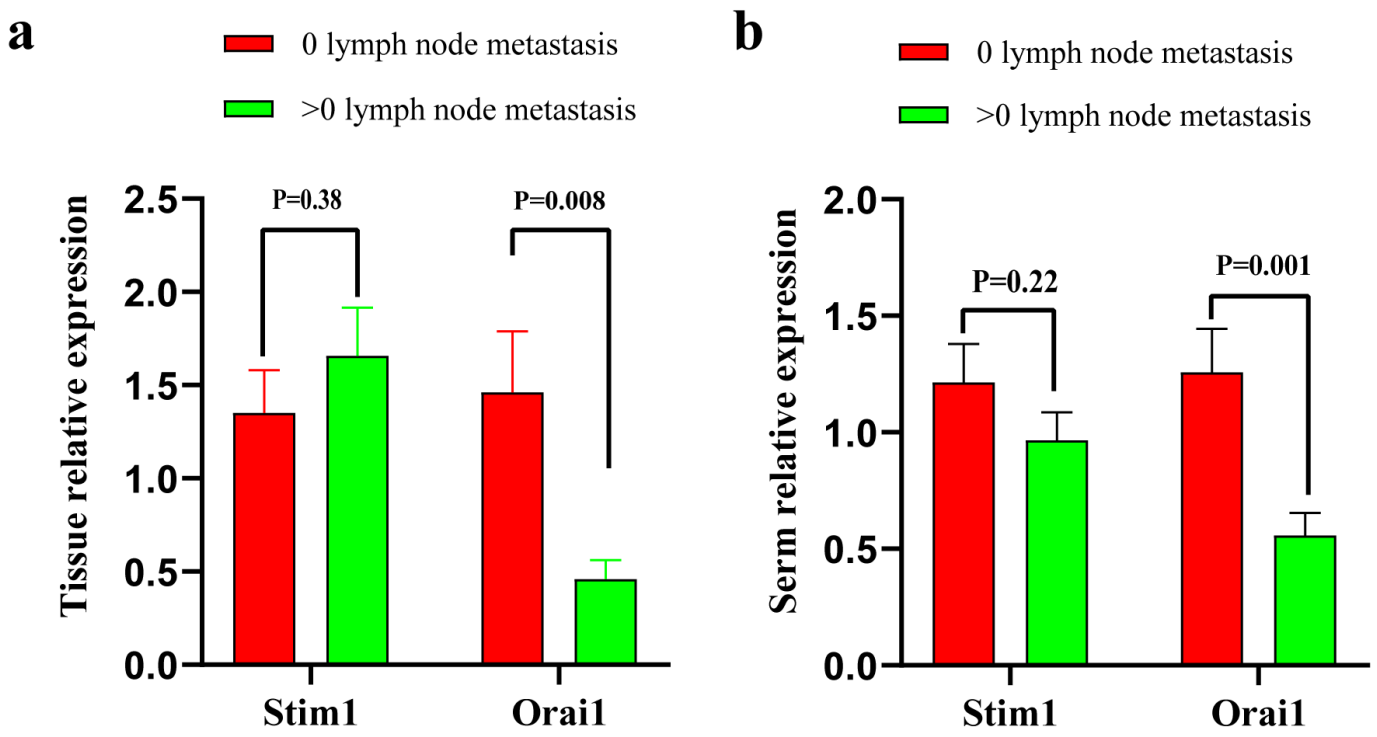

Figure 4

Comparison of Stim1 and Orai1 gene expression levels between the group without lymph node metastasis and the group with lymph node metastasis. a. Comparison of Stim1 and Orai1 genes in tumor tissue. b. Comparison of Stim1 and Orai1 in plasma.

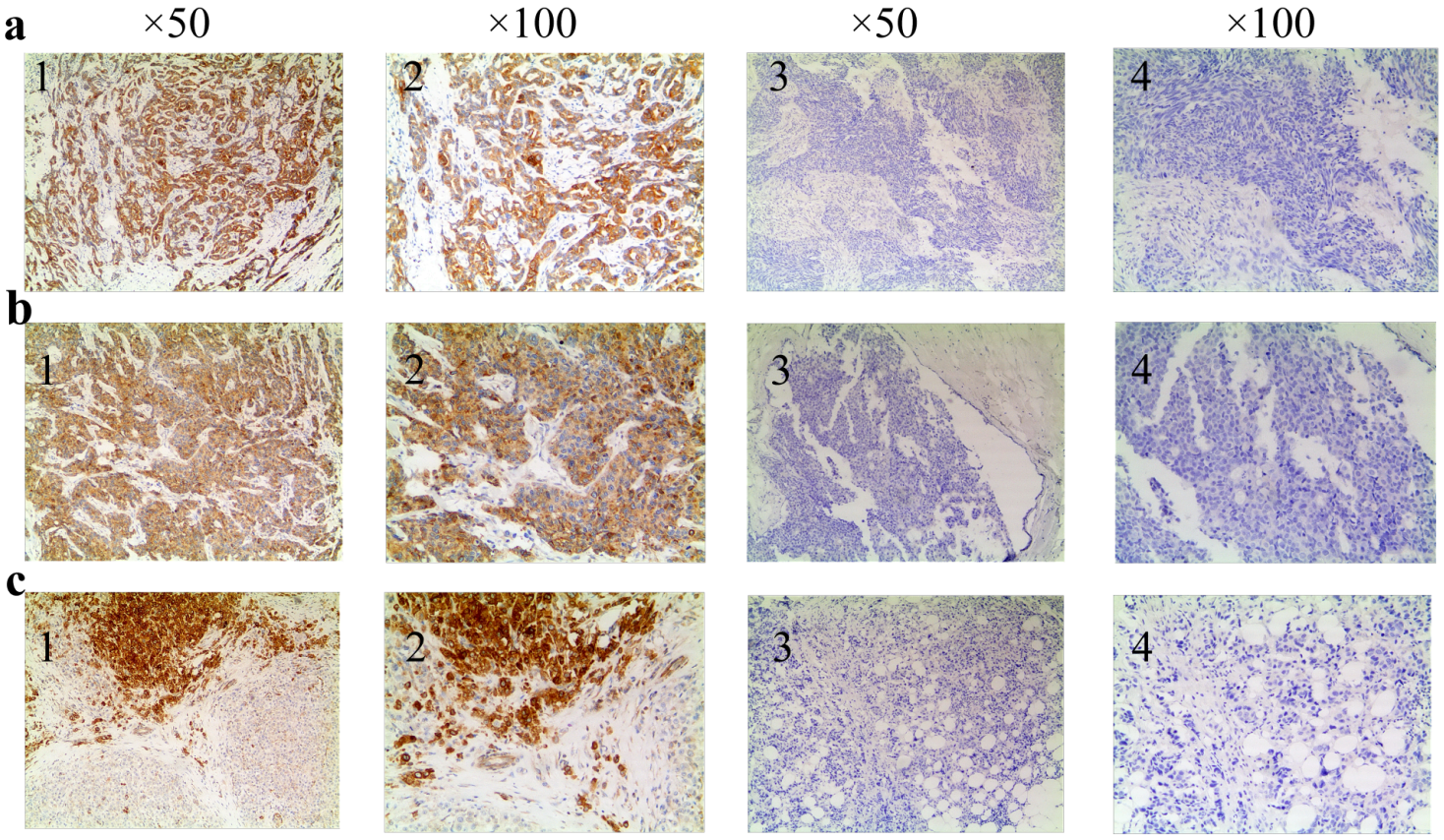

Figure 5

Stim1 immunohistochemistry. a: No lymph node metastasis group; b: 1-3 lymph node metastasis groups; c. ≥4 lymph node metastasis groups. 1. Stim1 high expression under 50x microscope; 2. Stim1 high expression under 100x microscope; 3. Stim1 low expression under 50x microscopy; 4: Stim1 low expression under 100x microscopy.

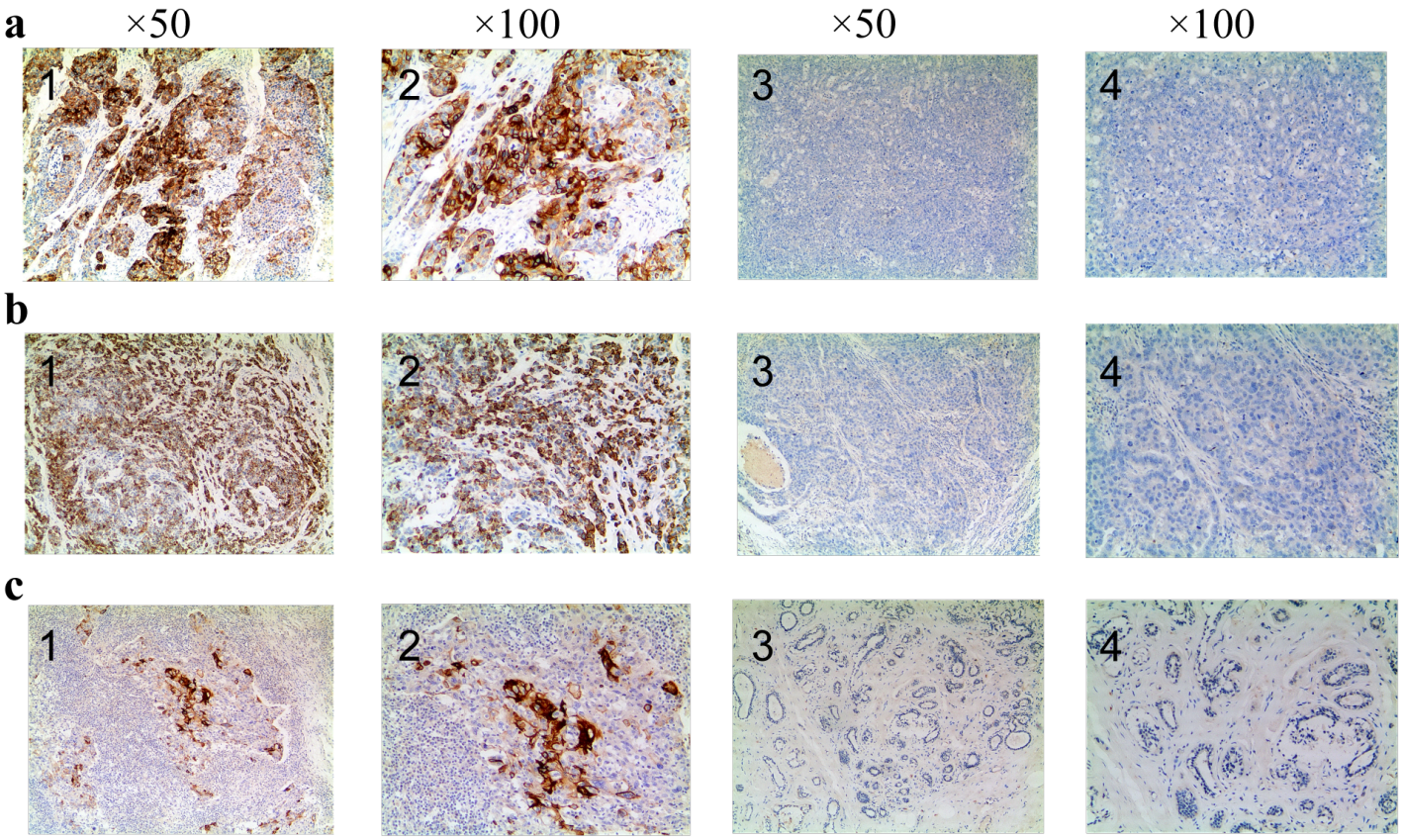

Figure 6

Orai1 immunohistochemistry. a: No lymph node metastasis group; b: 1-3 lymph node metastasis groups; c. ≥4 lymph node metastasis groups. 1. Orai1 high expression under 50x microscope; 2. Orai1 high expression under 100x microscope; 3. Orai1 low expression under 50x microscopy; 4: Orai1 low expression under 100x microscopy.

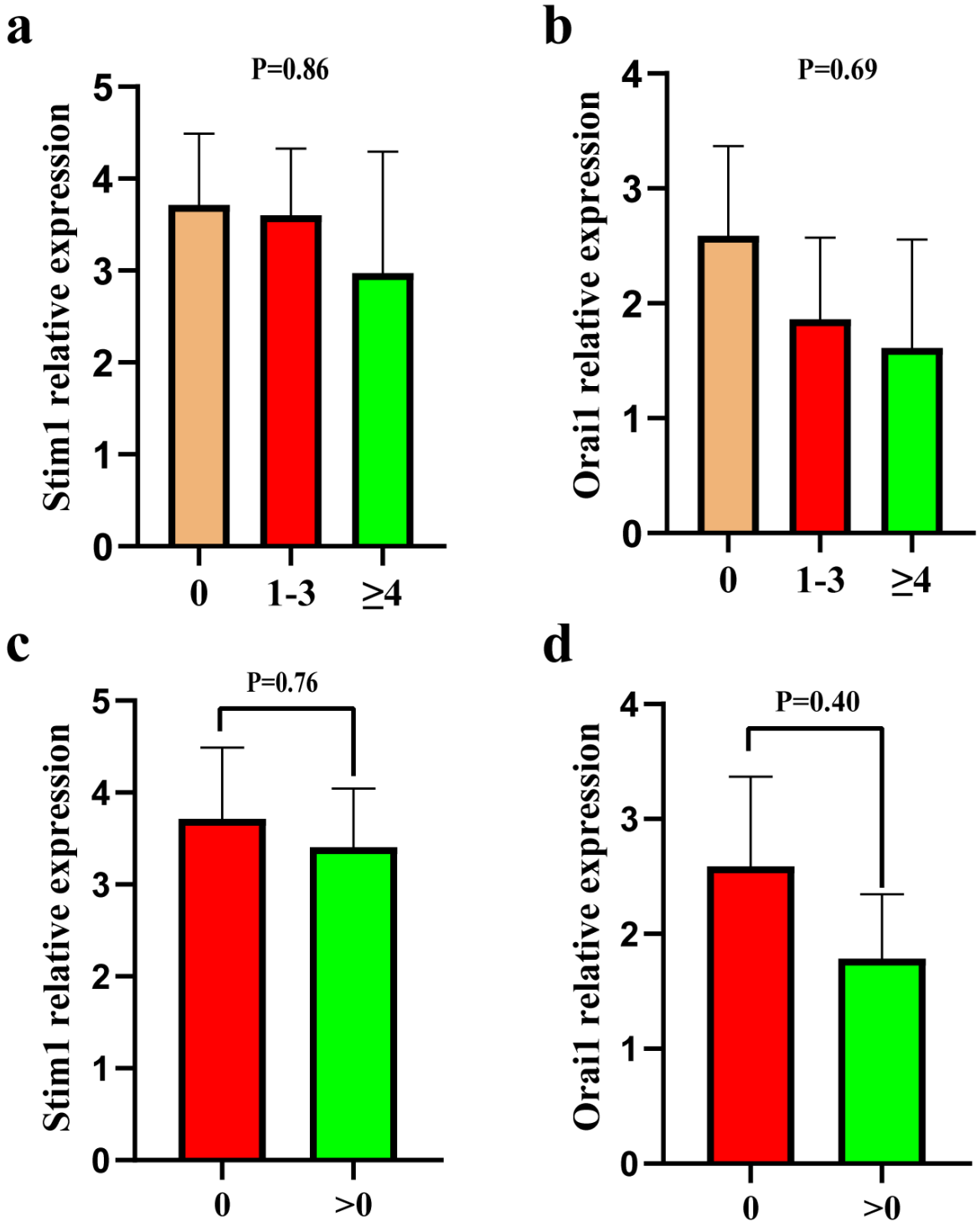

Figure 7

Stim1 and Orai1 protein expression. a. Comparison results of Stim1 among three groups of lymph node metastasis degree groups; b. Comparison results of Orai1 among three groups of lymph node metastasis degree groups. c. Comparison results of Stim1 between the group without lymph node metastasis and the group with lymph node metastasis; d. Comparison results of Orai1 between the group without lymph node metastasis and the group with lymph node metastasis. 0: No lymph node

metastasis group;1-3: 1-3 lymph node metastasis groups; ≥4≥4 lymph node metastasis groups; >0: Lymph node metastasis group.

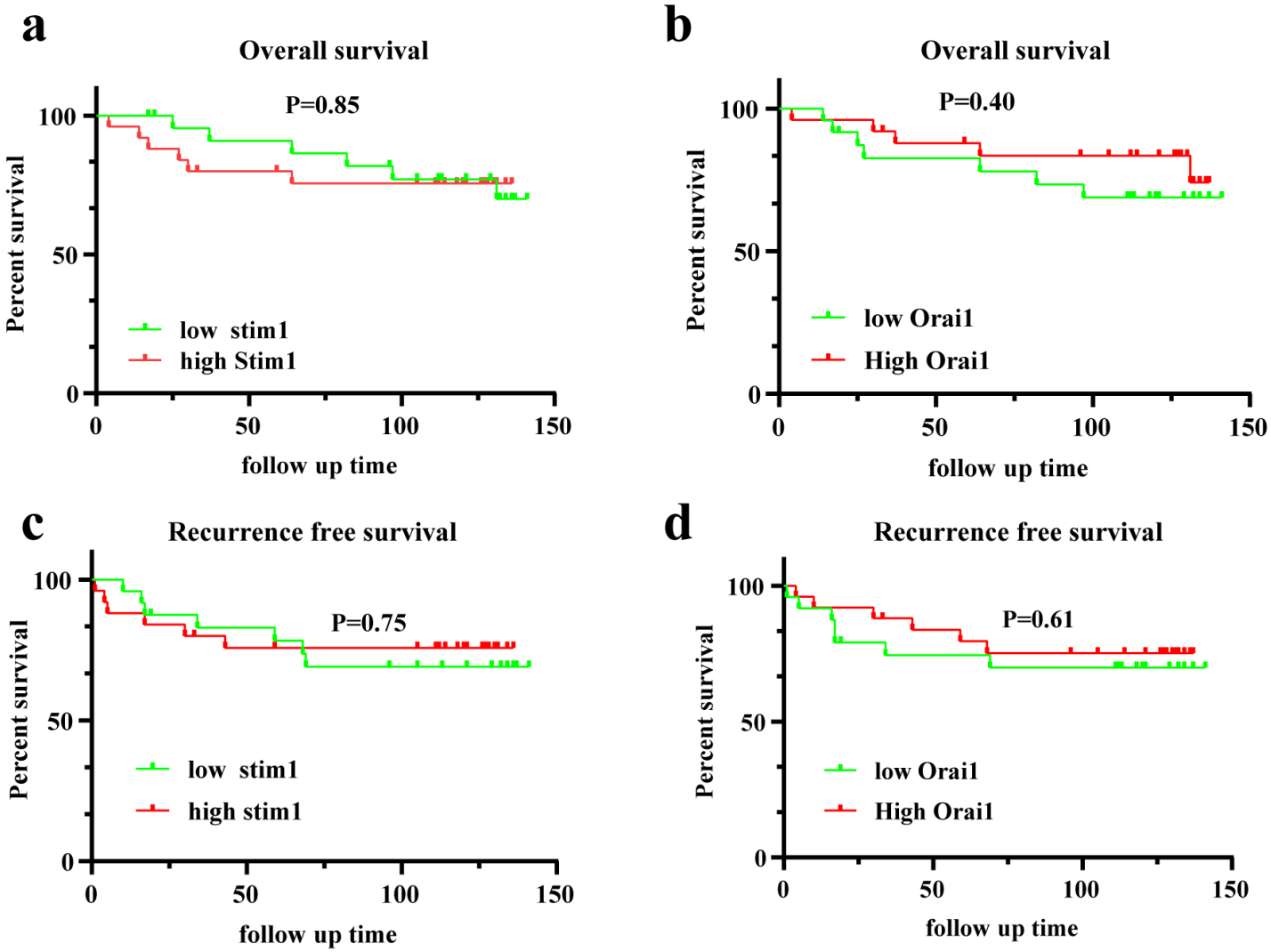

Figure 8

Survival analysis of Stim1 and Orai1. a. The effect of Stim1 expression level in tumor tissue on overall survival (OS); b. The effect of Orai1 expression level in tumor tissue on OS; c. The effect of Stim1 expression level in tumor tissue on recurrence free survival (RFS); d. The effect of Orai1 expression level in tumor tissue on RFS.

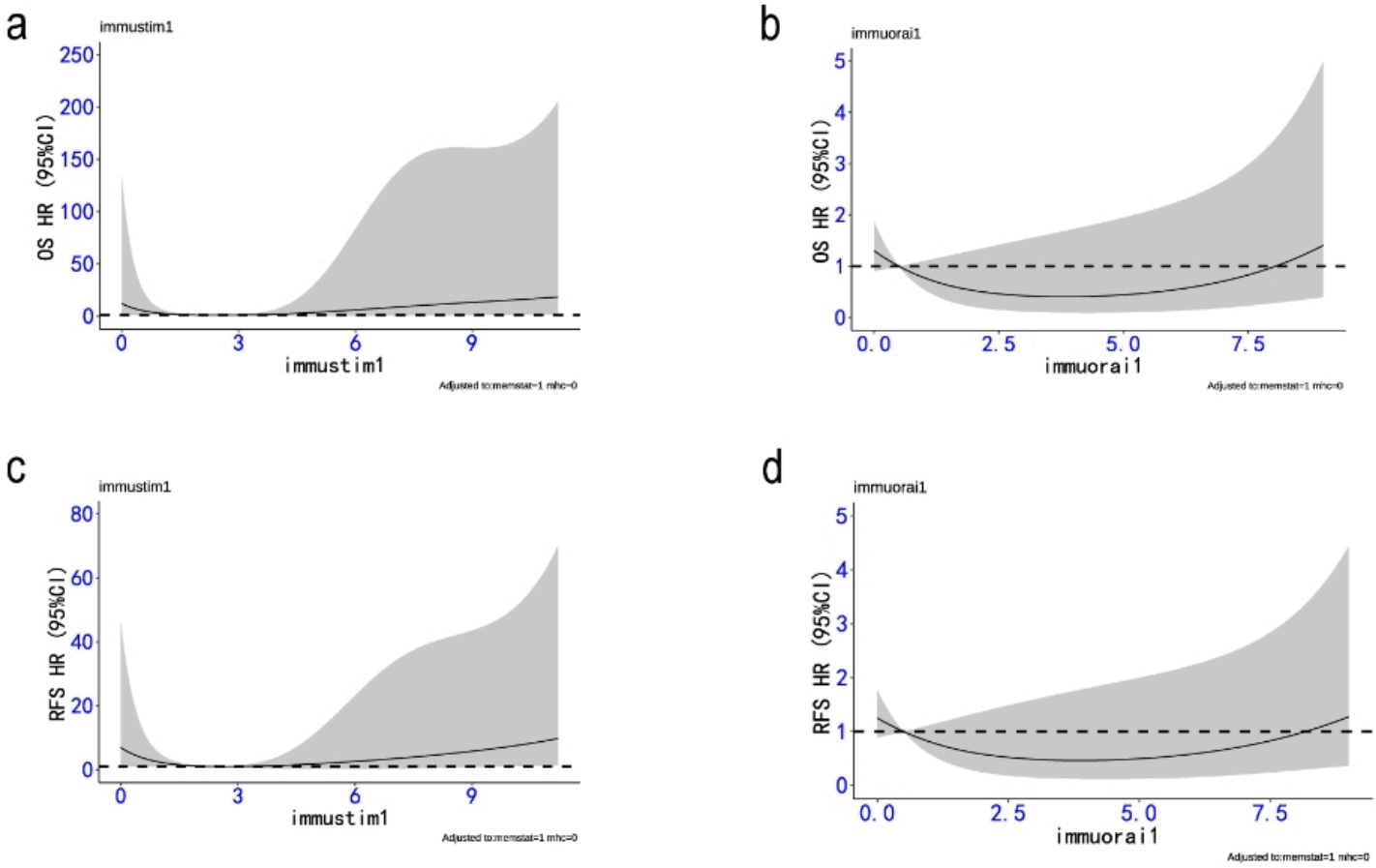

Figure 9

Restricted cubic spline (RCS) analysis of Stim1 and Orai1 expression levels on the survival of TNBC patients. Corrected for menstrual status and family history of breast cancer. HR=hazard ratio. (Due to the small sample size, P<0.1 was taken as a sign of statistical significance.)

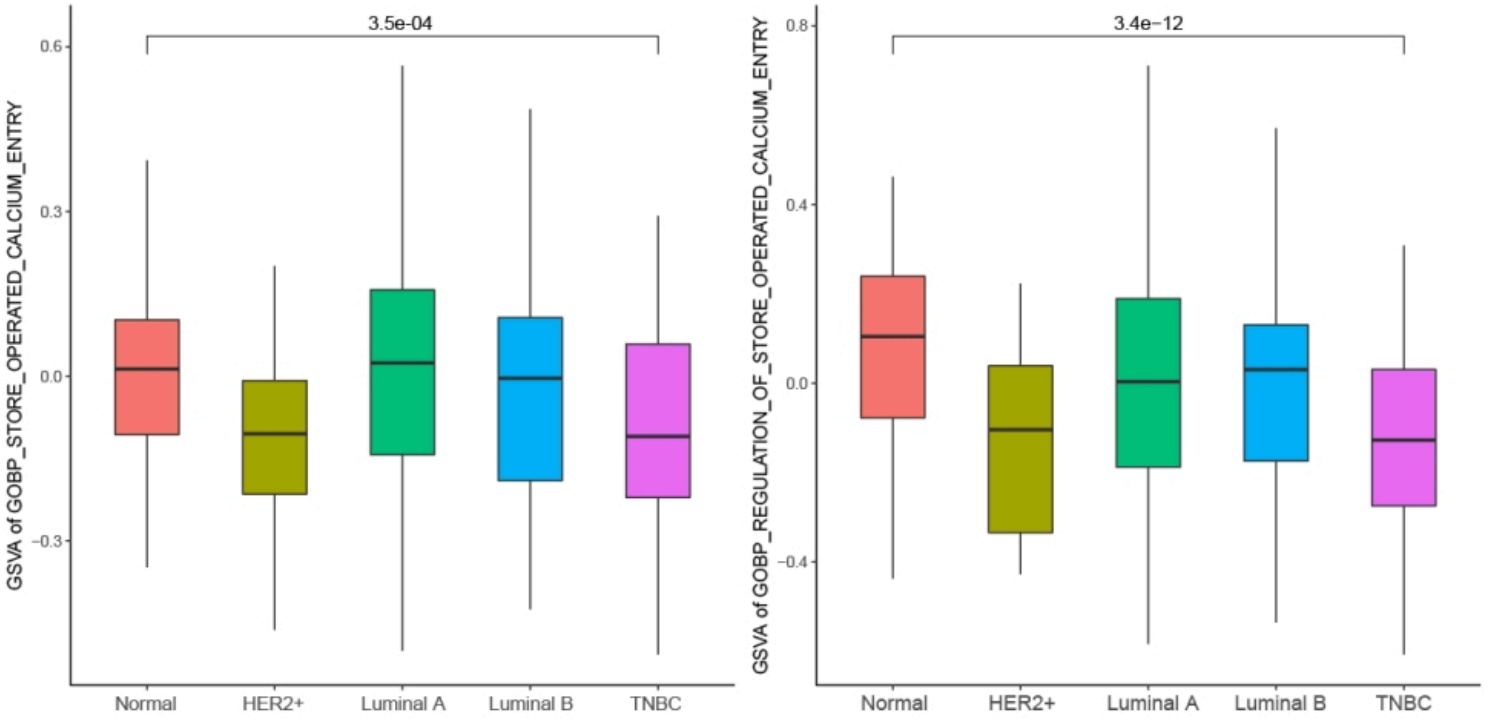

Figure 10

Differences in GSVA scores between two SOCE gene sets in the TCGA BRCA dataset samples

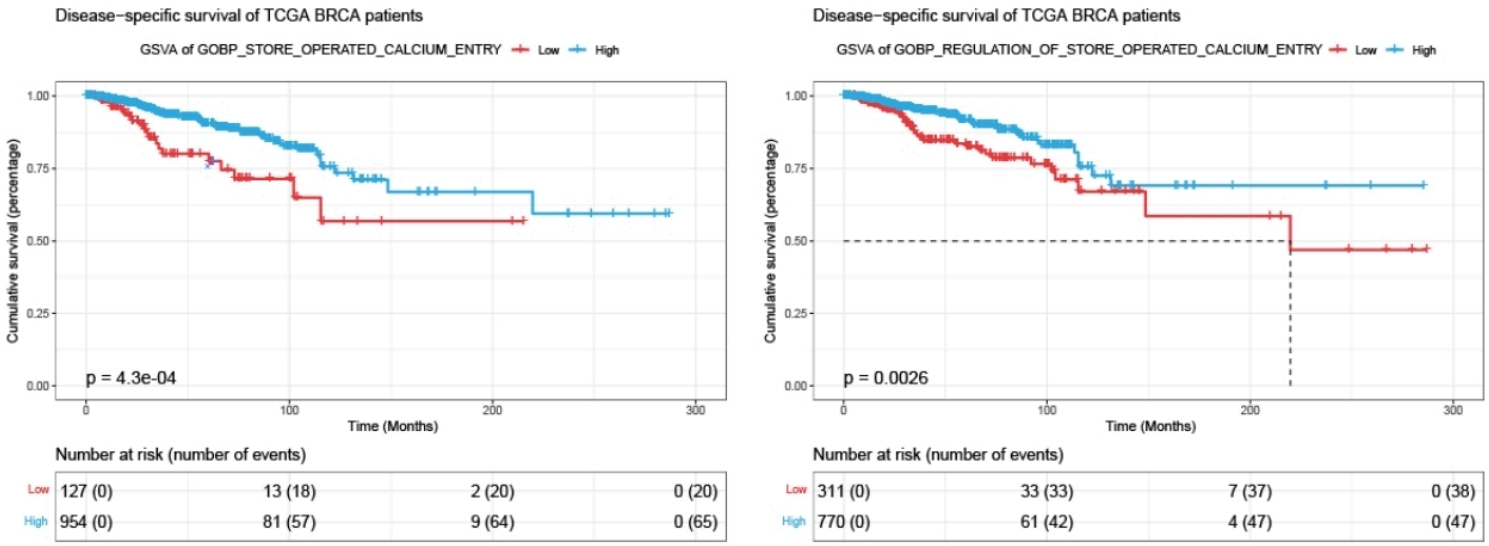

Figure 11

Disease specific survival (DSS) Kaplan-Meier (K-M) Curve of Two GSVA Scores in the TCGA BRCA Dataset

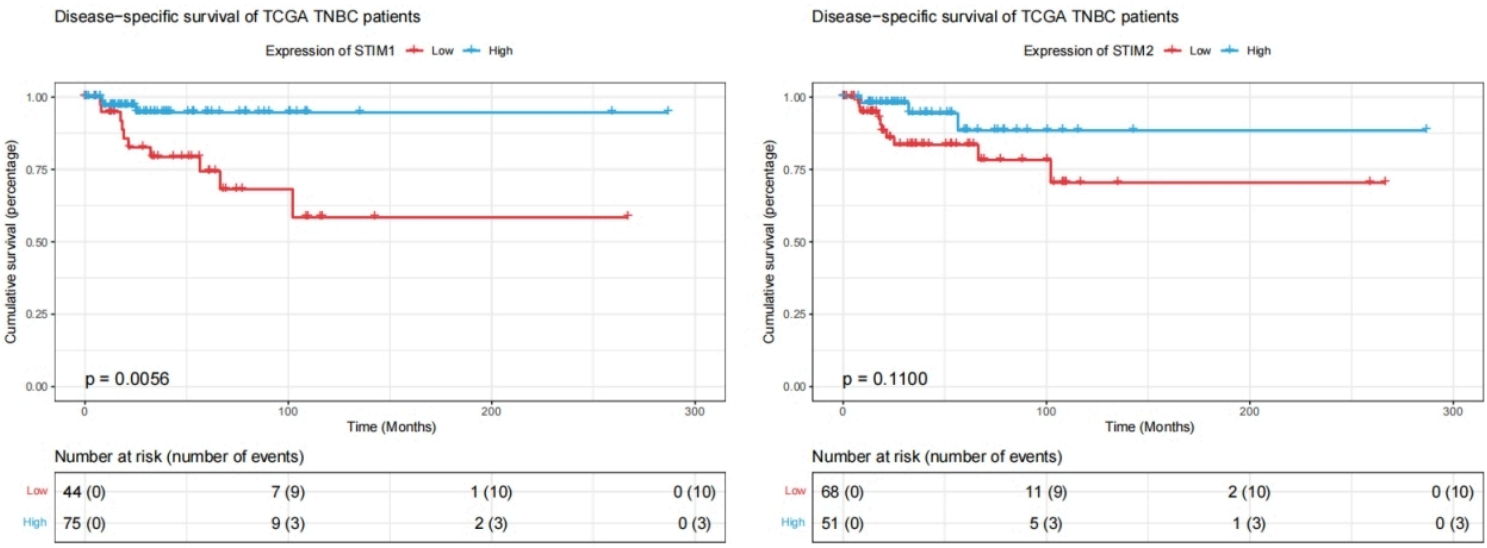

Figure 12

Disease specific survival (DSS) Kaplan-Meier (K-M) Curve of Stim1 and Stim2 Expressions in the TCGA BRCA Dataset

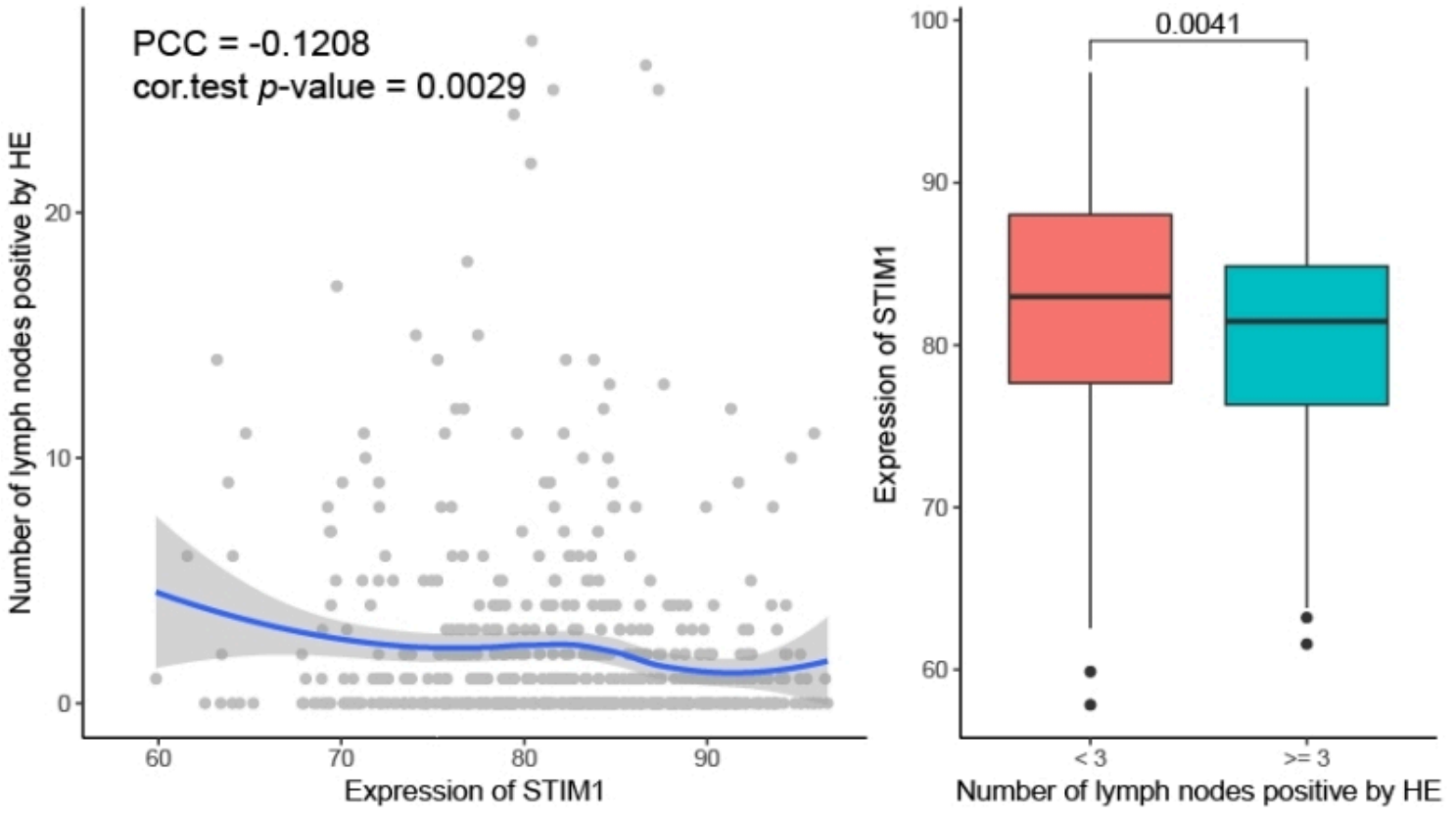

Figure 13

Stim1 expression is negatively correlated with the number of positive lymph nodes

Supplementary Files

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- SupplementaryTable.docx