Correlation Between Doppler Ultrasound Blood Flow Parameters and Angiogenesis and Proliferation Activity in Breast Cancer

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Source of support: Departmental sources

Background: The aim of this study was to assess the correlation between Doppler ultrasound blood flow parameters and angiogenesis and proliferation activity in breast cancer.

Material/Methods: We enrolled breast cancer patients (n=55) and benign tumor patients (n=40) from Tengzhou Central People's Hospital from Mar 2014 to Dec 2016. Doppler ultrasound examination was conducted to determine blood flow parameters, and immunohistochemistry (IHC) experiments were performed to determine the protein expression of angiogenesis genes, cell proliferation genes, and tumor-suppressor genes.

Results: Compared with benign tumors, the maximum velocity (Vmax) and resistance index (RI) were significantly different in I–II stage and III–IV stage breast cancer (P<0.01 and P<0.001, respectively). IHC assay showed that VEDGF165, NRP-1, SphK1, CD31, YAP, CTGF, and Gli2 proteins expressions were significantly higher in breast cancer patients (P<0.01 and P<0.001, respectively). PTEN and MFN2 protein expressions of breast cancer patients were significantly lower (P<0.01 or P<0.001, respectively) compared with those of benign tumor patients. VEDGF165, NRP-1, SphK1, CD31, YAP, CTGF, and Gli2 proteins expressions were positively correlated with Vmax and negatively correlated with RI in breast cancer. PTEN and MFN2 protein expressions were negative correlated with Vmax and positively correlated with RI in breast cancer patients.

Conclusions: Decreased RI and increased Vmax are correlated with angiogenesis, proliferation, and tumor suppression in breast cancer.

MeSH Keywords: **Cell Proliferation • Genes, Tumor Suppressor • Nuclear Receptor Coactivator 3 • Saralasin**

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/914395

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Background

Breast cancer is one of the most common malignant tumors among women. Although there are currently many treatments, breast cancer is often detected at an advanced stage, which results in poor outcome [1–3]. Clinical diagnosis of breast cancer is based on the size of the patient's clinically palpated mass. Diagnosis lacks objectivity and is limited; pathological examination is the criterion standard for diagnosis of breast cancer, but the pathological findings must be obtained after surgical resection, which causes damage to the body. Therefore, imaging evaluation of breast cancer has drawn increasing attention from scholars at home and abroad. The formation of neovascularization in breast cancer lesions, the decrease in blood flow resistance, and the increase of blood flow are the basis of cancer cell growth. Accurately assessing the blood flow status in tumor lesions can provide a basis for judging the malignancy of tumors [4,5]. Doppler ultrasound is a commonly used method for clinical assessment of blood flow status. Quantitative measurement of blood flow parameters can quantify blood flow in local tissues. It has been reported that Vmax of ultrasound blood flow parameters in tumor lesions is significantly increased and RI is significantly reduced, but the relationship between Vmax and RI and the degree of malignancy of breast cancer is not clear [6]. In our present study, we analyzed the correlation between Vmax and RI, which are Doppler ultrasound flow parameters, and angiogenesis and cancer cell proliferation in breast cancer.

Material and Methods

Clinical samples

We enrolled breast cancer patients (n=55), including I–II stage (n=24) and III–IV stage (n=31), and benign tumor patients (n=40) from Tengzhou Central People's Hospital from Mar 2014 to Dec 2016. All the patients underwent puncture pathological diagnosis. Benign tumor patients were 38~72 years old, with a median age of 57 years old. I–II stage breast cancer patients were 37~59 years old with BMI (22.9±3.5) kg/m^2 and III–IV stage breast cancer patients were 34~58 years old with BMI (23.1±3.2) kg/m^2. There was no statistically significant difference in patient information between clinical samples from different sources (P>0.05). The study was approved by the Ethics Committee of Tengzhou Central People's Hospital, and all patients signed informed consent.

Detection method of Doppler ultrasound blood flow parameters

For diagnosis, we used an Acuson Antares Doppler ultrasound device (Siemens, Germany) with probe frequency 7.5~13 MHz.

First, the location of the lesion was confirmed by routine scanning, and then the section with the richest blood flow signal was selected for blood flow parameter measurement. The angle between the sound beam and the blood stream was less than 30°, measuring maximum blood flow velocity (Vmax) and resistance index (RI).

IHC assay

The tissues of breast cancer and benign tumor patients were fixed in 4% paraformaldehyde, paraffin-embedded, and sliced into sections. The sections were dewaxed and hydrated using 3% hydrogen peroxide to culture in an incubator for 30 min. Proteins were repaired by microwave repair and washed with PBS 3 times. We added primary antibodies (VEGF, NPR1, SphK1, CD31, YAP, CTGF, Gli2, PTEN and MFN2; Abcam, UK) at 1: 200 to culture at 4°C overnight. After washing 3 times with PBS, we added the secondary antibodies for culturing at 37°C for 30 min, followed by washing with PBS 3 times, using DAB staining, followed by washing, hematoxylin staining, and dehydration with a transparent seal. We used Image-pro image analysis software (Version 6.0, Media Cybernetic, USA) to assess the IOD value of different tissues.

Statistical treatment

Measurement data are shown as mean ±SD (standard deviation). Multigroup comparisons were made using one-way ANOVA with LSD test. All data were analyzed using SPSS 19.0 software. Pearson correlation analysis was performed. P<0.05 was set as the level of statistical significance.

Results

Ultrasonic blood flow parameters of breast cancer and benign tumor patients

We assessed Doppler ultrasound blood flow parameters Vmax and RI in the I–II stage breast cancer group, the III–IV stage breast cancer group, and the benign tumor group. We found that Vmax in the of stage I–II breast cancer group and III–IV stage breast cancer group were significantly higher compared with that of the benign tumor group (P<0.01 or P<0.001). The RI of the I–II stage group and III–IV stage group were significantly lower compared with that of the begin tumor group (P<0.01 or P<0.001). The data are shown in Figure 1.

Angiogenesis proteins in breast cancer and benign tumors and correlation with Vmax and RI in breast cancer

Compared with the benign tumor group, the levels of VEGF165, NPR1, SphK1, and CD31 proteins in breast cancer (I–II stage
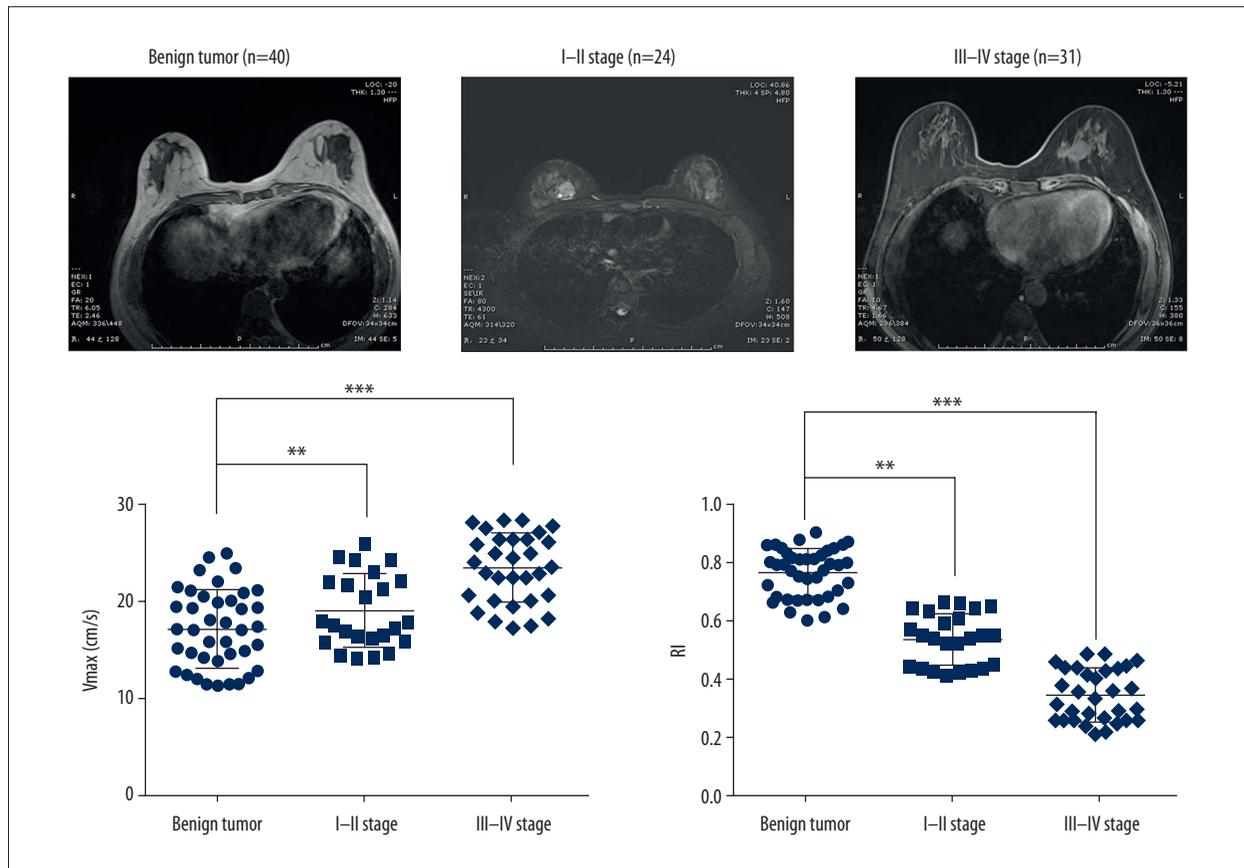
Figure 1. Doppler ultrasound blood flow parameters. ** P<0.01 *vs.* benign tumor; *** P<0.001 *vs.* benign tumor.

and III–IV stage) were significantly higher (P<0.01 or P<0.001, respectively). Vmax was negatively correlated with VEGF165, NPR1, SphK1, and CD31 expression in breast cancer (P<0.05, respectively), and RI was positively correlation with VEGF165, NPR1, SphK1, and CD31 in breast cancer (P<0.05, respectively). The data are shown in Figure 2.

Proliferation proteins in breast cancer and benign tumors and correlation with Vmax and RI in breast cancer

Compared with the benign tumor group, the levels of YAP, CTGF, and Gli2 proteins in the breast cancer groups (I–II stage and III–IV stage) were significantly higher (P<0.01 or P<0.001, respectively). Vmax was negatively correlated with YAP, CTGF, and Gli2 levels in the breast cancer groups (P<0.05), and RI was positively correlated with YAP, CTGF, and Gli2 in breast cancer (P<0.05, respectively). The data are shown in Figure 3.

Cancer-suppressor proteins in breast cancer and benign tumors and correlation with Vmax and RI in breast cancer

Compared with the benign tumor group, the expression of PTEN and MFN2 proteins in breast cancer (I–II stage and III–IV stage) were significantly higher (P<0.01 and P<0.001, respectively).

Vmax was positively correlated with PTEN and MFN2 in breast cancer (P<0.05, respectively), and RI was positively correlated with PTEN and MFN2 in breast cancer (P<0.05). The data are shown in Figure 4.

Discussion

Angiogenesis is an important characteristic of breast cancer, and it is also the pathological basis of abnormal proliferation and invasion of cancer cells. Neovascularization of tumor vessels is characterized by rougher muscle structure, high permeability of the vessel wall, and more arteriovenous short circuits, which can provide abundant blood flow to the lesion [7–9]. Color Doppler ultrasound is a noninvasive examination method used in clinical evaluation of tissue blood perfusion characteristics. It can quantitatively evaluate blood flow characteristics through Vmax, RI, and other parameters [10,11]. In the present study, the analysis of color Doppler ultrasound blood flow parameters Vmax and RI of breast cancer lesions showed that Vmax was significantly higher and RI was significantly lower in the breast cancer groups compared with the benign tumor group, and Vmax and RI were significantly associated with increasing stage. These results suggest that breast cancer lesions have significantly reduced blood
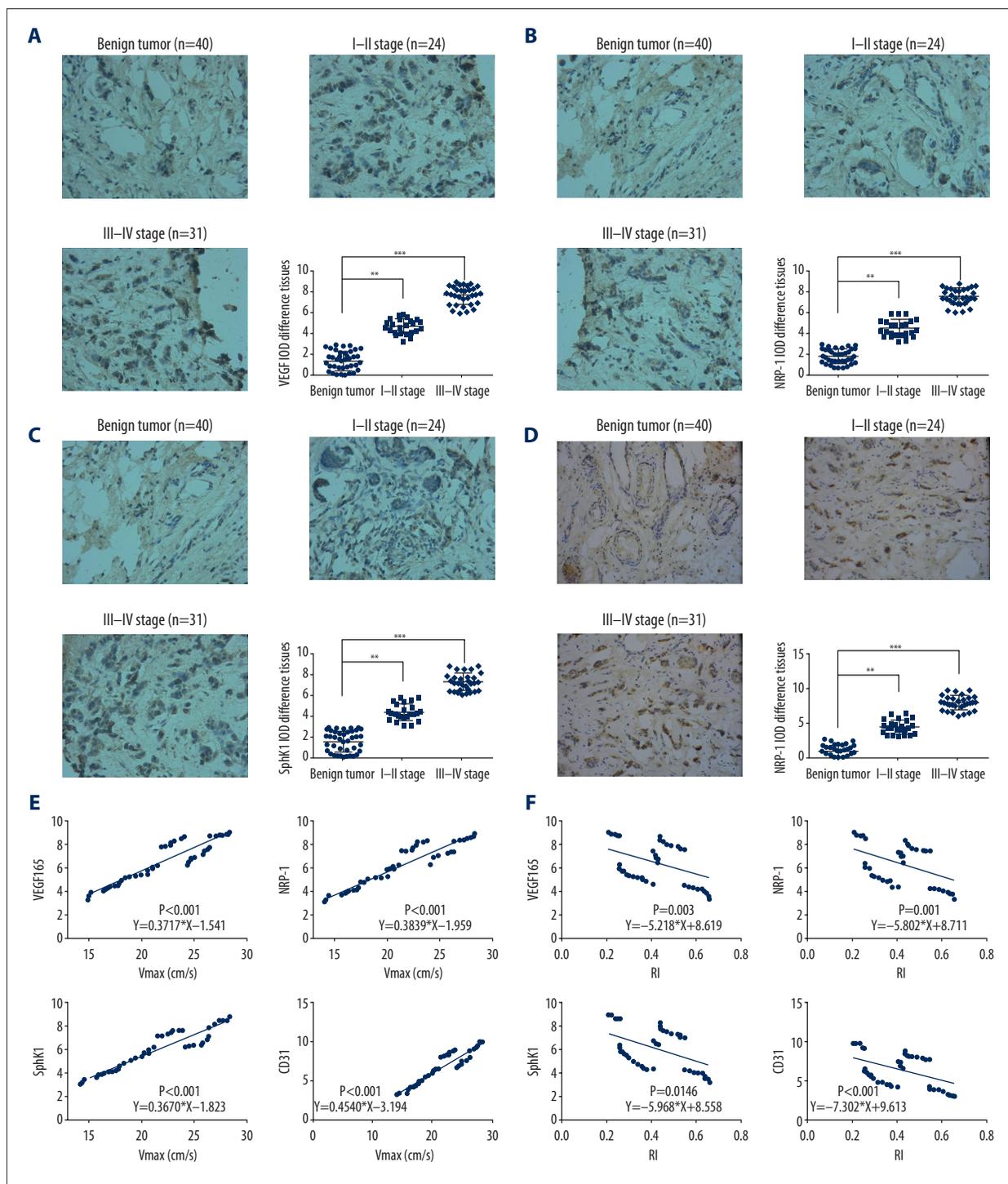
Figure 2. VEGF165, NRP-1, and SphK1 proteins expressions and correlation with Vmax or RI (×200). (**A**) VEGF165 protein expression in different tissues by IHC (×200). ** P<0.01 *vs.* benign tumor; *** P<0.001 *vs.* benign tumor. (**B**) NRP-1 protein expression in different tissues by IHC (×200). ** P<0.01 *vs.* benign tumor; *** P<0.001 *vs.* benign tumor. (**C**) SphK1 protein expression in different tissues by IHC (×200). ** P<0.01 *vs.* benign tumor; *** P<0.001 *vs.* benign tumor. (**D**) VEGF165, NRP-1, and SphK1 correlation with Vmax in breast cancer. (**E**) VEGF165, NRP-1, and SphK1 correlation with RI in breast cancer.
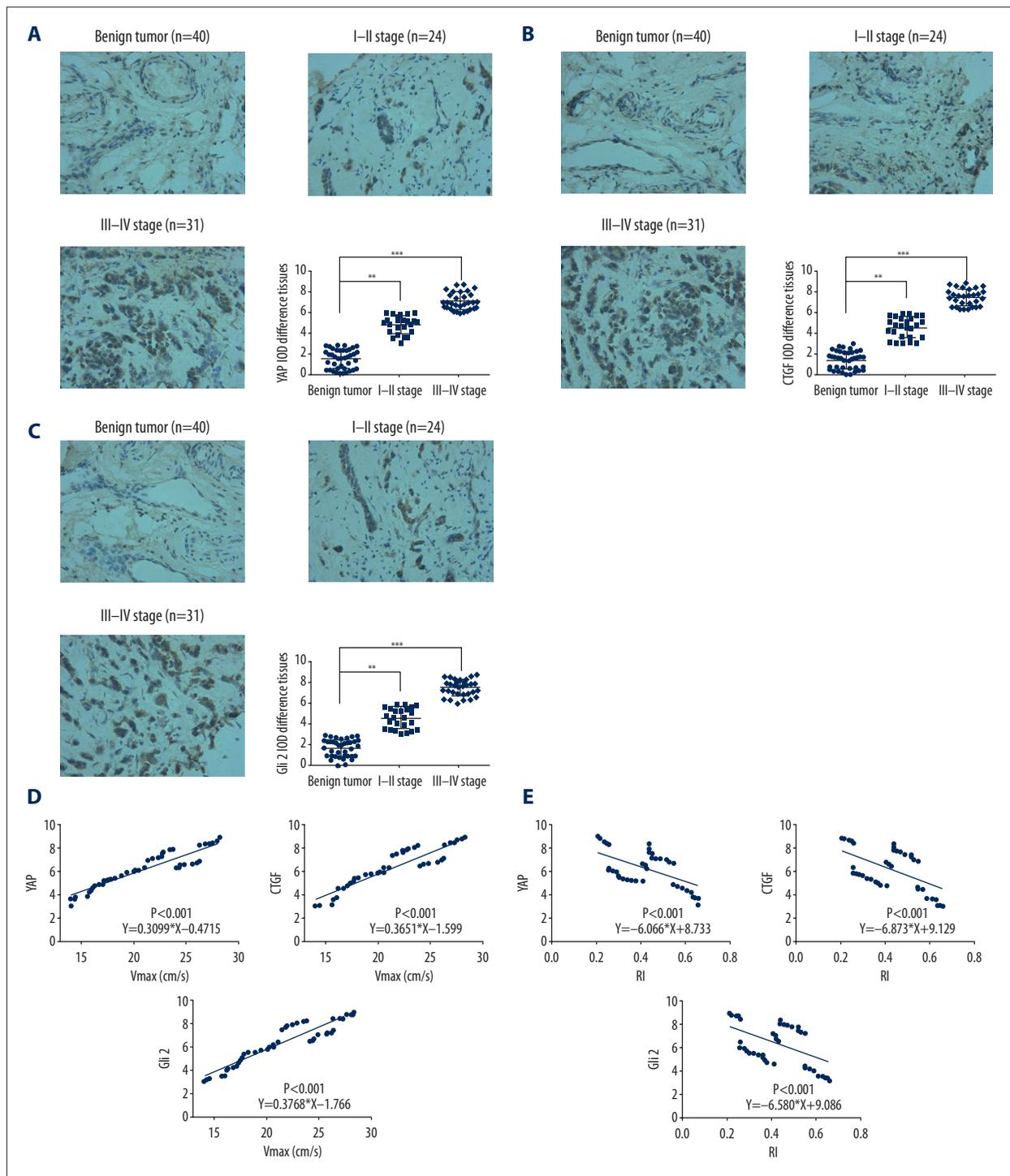
Figure 3. YAP, CTGF and Gli2 proteins expressions and correlation with Vmax or RI (×200). (**A**) YAP protein expression in different tissues by IHC (×200). ** $P<0.01$ *vs.* benign tumor; *** $P<0.001$ *vs.* benign tumor. (**B**) CTGF protein expression in different tissues by IHC (×200). ** $P<0.01$ *vs.* benign tumor; *** $P<0.001$ *vs.* benign tumor. (**C**) Gli2 protein expression in difference tissues by IHC (×200). ** $P<0.01$ *vs.* benign tumor; *** $P<0.001$ *vs.* benign tumor. (**D**) YAP, CTGF, and Gli2 correlation with Vmax in breast cancer. (**E**) YAP, CTGF, and Gli2 correlation with RI in breast cancer.
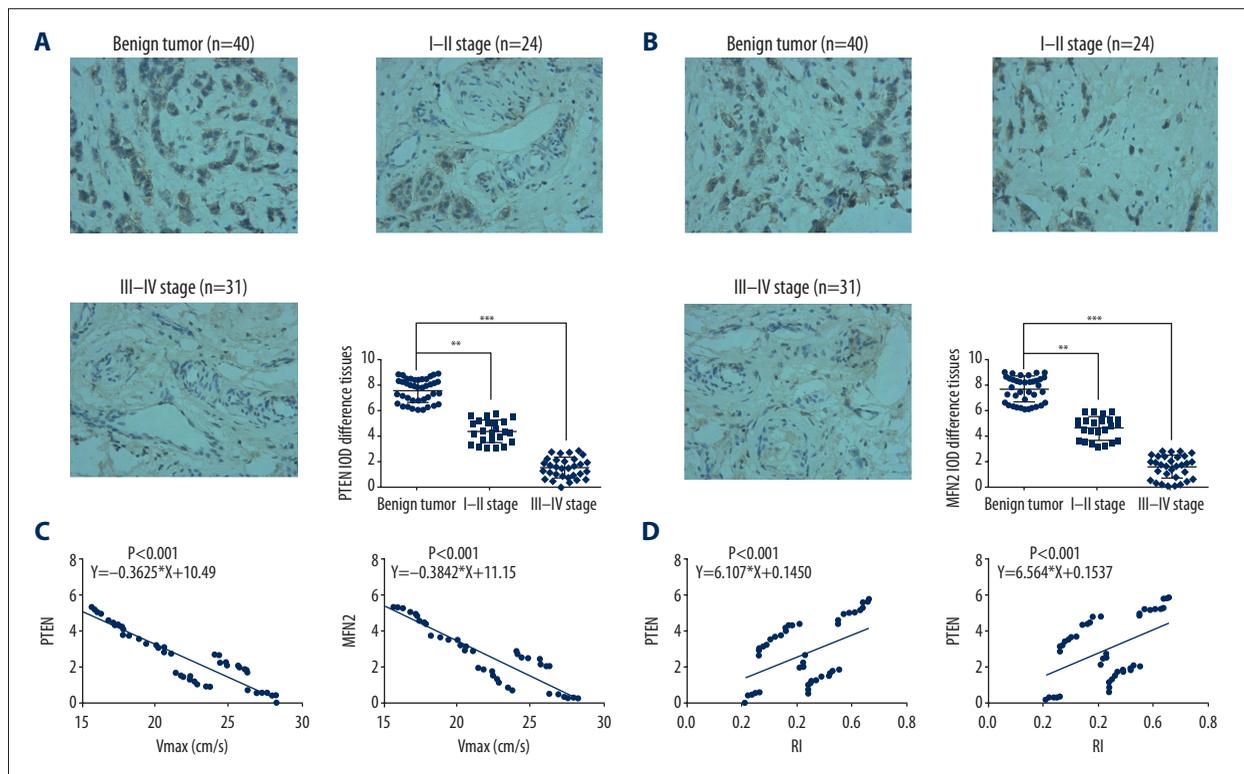
Figure 4. PTEN and MFN2 proteins expressions and correlation with Vmax or RI (×200). (**A**) PTEN protein expression in different tissues by IHC (×200). ** $P<0.01$ *vs.* benign tumor; *** $P<0.001$ *vs.* benign tumor. (**B**) MFN2 protein expression in different tissues by IHC (×200). ** $P<0.01$ *vs.* benign tumor; *** $P<0.001$ *vs.* benign tumor. (**C**) PTEN and MFN2 correlation with Vmax in breast cancer. (**D**) PTEN and MFN2 correlation with RI in breast cancer.

flow resistance and significantly higher blood flow velocity, and is associated with higher tumor stage. The further reduction of blood flow resistance and the marked increase in blood flow velocity are associated with the structure of new blood vessels within the tumor lesion, and promote the growth of breast cancer lesions through blood perfusion.

Tumor angiogenesis is the pathological basis of decreased blood flow resistance and increased blood perfusion. VEGF165 is an important member of the VEGF family, with receptors on endothelial cells and tumor cell membranes. NPR-1, after combination, promotes endothelial cells tube structure, and can promote the growth of tumor cells [12–16]. SphK1 catalytic sphingosine phosphorylation of SIP is an important catalytic enzyme process. SIP, after combining with the membrane surface SIPR, can initiate downstream MAPK and PI3K/AKT signaling, which promotes endothelial cell proliferation and migration and formation of tube-type structures [17,18]. CD31 is the vascular tight junction marker, and CD31 overexpression in vessels may play a key role in cancer development [19,20]. Under the combined action of VEGF165 and S1P, the formation of new blood vessels in breast cancer lesions is significantly increased. To assess the role of VEGF165 in breast lesions and to determine how S1P angiogenesis is mediated, we

analyzed the angiogenic gene expression levels. The results showed that VEGF165, NRP-1, SphK1, and CD31 proteins expressions in the breast cancer groups were significantly higher compared with those of benign tumor patients; however, VEGF165, NRP-1, SphK1, and CD31 proteins levels in the III–IV stage group were higher than those of the I–II stage breast cancer group. Further analysis of the correlation between angiogenesis gene expression and blood flow characteristics showed the following: Vmax was positively correlated with VEGF165, NRP-1, SphK1, and CD31 proteins expressions and RI was negatively correlated with VEGF165, NRP-1, SphK1, and CD31 proteins expressions in breast cancer patients. This indicates that vascular neovascularization mediated by VEGF165, NRP-1, SphK1 and CD31 in breast cancer can reduce blood flow resistance and increase blood perfusion.

In breast cancer lesions, increased angiogenesis and blood flow can create a favorable local environment for cancer cell proliferation and affect expression of multiple proliferation genes. YAP is a downstream gene of the Hippo pathway that suppresses cancer development. Hippo signaling pathway deactivation can lead to YAP protein transfer from cytoplasm to nucleus and storage in the nucleus, after which YAP stimulates CTGF and Gli2, which were target genes of YAP, and increases cancer cell

proliferation [21–24]. By analyzing the differences in expressions of YAP, CTGF, and Gli2 proteins in different stages of breast cancer, we found that YAP, CTGF, and Gli2 proteins were significantly higher in breast cancer patients compared with those in patients with benign tumors. We also found that YAP, CTGF, and Gli2 proteins expression in the III–IV stage group were higher than in the I–II stage breast cancer group. The correlation between cell proliferation gene expression and blood flow characteristics was further analyzed, showing that Vmax was positively correlated with YAP, CTGF, and Gli2, and showing that RI was negative correlated with YAP, CTGF, and Gli2 in breast cancer. These results confirm that decreasing blood flow resistance and increasing blood flow velocity can increase cancer cells proliferation mediated by YAP, CTGF, and Gli2 in breast cancer.

In the breast cancer pathological process, the abnormal proliferation of cancer cells is associated with high expression of proliferation genes and low expression of tumor-suppressor genes. PTEN can suppress the PI3K/AKT pathway to inhibit cell proliferation and induce cell apoptosis [25,26]. MFN2 also reduces cell proliferation by inhibiting the ERK1/2/MAPK pathway via regulation of Ras expression [27,28]. In our present study, we found that expression of PTEN and MFN2 in breast cancer patients was significantly lower than in patients with benign tumors. We also found that increasing stage of breast cancer was associated with lower expression of PTEN and MFN2. Further analysis showed that Vmax was negatively correlated with PTEN and MFN2 expression and RI was positively correlated with PTEN and MFN2 expression in breast cancer patients.

Conclusions

Doppler ultrasound blood flow parameters, including changes in Vmax increase and RI decrease, were assessed, along with expression of cancer-related proteins (VEGF165, NRP-1, SphK1, CD31, YAP, CTGF, Gli2, PTEN, and MFN2) in breast cancer. Our research also explains the mechanisms of Doppler ultrasound blood flow parameters, such as changes in different stages of breast cancer.

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