Original Article

Correlation of elastic characteristics of breast lesions with the expression of Smad2/3

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Background: To analyze the relationship between elastic characteristics and the expression of Smad2/3 in breast lesions.

Methods: Between April 2018 and October 2018, 135 lesions from 130 patients who underwent shear wave elastography before surgical excision or biopsy were included in the study. The shear wave elasticity features of the lesions, expression of Smad2/3 of the specimens, and their combined diagnostic efficacy was analyzed.

Results: Of the 135 lesions, 51 were malignant and 84 were benign. The elasticity ratio of lesions to peripheral parenchyma, maximum elasticity, mean elasticity, prevalent rate of "stiff rim sign", and the expression level of Smad2/3 in the malignant pathological changes were obviously superior to those with benign pathological change (P<0.001). The Smad2/3 expression level had a positive correlation with the maximum, average elasticity, and the elastic ratio of lesions to peripheral parenchyma (r=0.657, 0.640, and 0.470, respectively, P<0.001). The expression of Smad2/3 in lesions with "stiff rim sign" was statistically higher than that in lesions without "stiff rim sign" (P<0.001). Moreover, the combination of Smad2/3 expression and "stiff rim sign" was shown to greatly raise the sensitivity (100%) and accuracy (94.56%) in the differential diagnosis of mammary gland disease.

Conclusions: In light of the findings, maximum, mean elasticity, elasticity ratio of lesions to peripheral parenchyma, and "stiff rim sign" are correlated with the expression of Smad2/3. The combination of the expression of Smad2/3 and "stiff rim sign" might contribute to the diagnosis of breast carcinoma.

Keywords: Breast neoplasms; shear wave elasticity; Smad2/3

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Introduction

Breast carcinoma is the primary cause of cancer mortality in females, with an estimated 1 in 8 women developing the disease at some point in their lives (1). Evidence suggests that increased matrix stiffness is a hallmark of breast cancer (2). As an ideal imaging technology based on tissue stiffness, shear wave elasticity (SWE) has been used to quantitatively and qualitatively evaluate the tissue elasticity, showing high sensitivity, specificity, and accuracy in differentiating benign and malignant breast lesions (3).

Transforming growth factor-β (TGF-β), a cytokine, participates in antitumor activity, such as proliferation inhibition, but also in carcinogenic processes, such as growth stimulation, increased movement, invasion, and

metastasis (4,5). Our previous studies have shown that TGF-β is associated with maximum elasticity, mean elasticity, the elasticity ratio of lesions to peripheral parenchyma (5). The 2 members of the Smad family, Smad2 and the analogous protein Smad3, mediate the signal transduction of TGF-β (6). Further, TGF-β binds to transmembrane serine threonine kinase receptors, causing downstream phosphorylation and activation of Smad2 and Smad3, which are then transferred to the nucleus to control the transcription of target genes (7,8). Data from several studies has suggested that the expression of Smad2/3 in malignant tumors is closely related to neoplasm progression, metastasis, and poor prognosis (9). Moreover, several lines of evidence have previously linked Smad2/3 to epithelial-mesenchymal transition (EMT) and vasculogenic mimicry (VM) formation in breast cancer (10,11). Both Smad2 and Smad3 are also known to control the expression of a number of dermal extracellular matrix (ECM) components, including collagen and fibronectin (12,13). In previous research, collagen and elastic fiber content were shown to be closely related to the elastic characteristic of breast diseases, which may have a pivotal role in stiffness of breast neoplasm (14,15).

Nevertheless, although a number of cross-sectional studies have suggested that abnormal expression of Smad2/3 can affect the occurrence and development of breast cancer, no study has proved whether Smad2/3 is related to the elastic characteristics of breast lesions. Thus, this study aimed to investigate the relationship between shear wave elasticity and the expression of Smad2/3 in breast lesions. We present the following article in accordance with the STARD reporting checklist (available at https://dx.doi.org/10.21037/atm-21-5627).

Methods

Patients

From April 2018 to October 2018, a total of 130 women with 135 consecutive breast lesions were recruited to the study, of which 5 patients had 2 masses. The average age of participants was 44 years (18–73 years). The maximum diameter of the lesion was 0.4 to 5.5 cm (mean diameter ± normalized, 1.6±0.8 cm). All lesions were examined by shear wave elastography (SWE), and the pathology was confirmed by surgery.

The inclusion criteria were as follows: (I) pathological findings were available and definite, (II) patients underwent surgery. Patients were excluded if they met the following criteria: (I) had received neoadjuvant chemotherapy or radiotherapy, (II) lacked clinical, pathological, and prognostic follow-up data.

A protocol was prepared before the study without registration. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The prospective study was approved by the Ethics Committee of the Chinese PLA General Hospital (No. S2020-336-01) and informed consent was taken from all the patients.

SWE examinations

The Aixplorer® ultrasonic diagnostic system (SuperSonic Imagine, Aix-en-Provence, France), with a transducer frequency of 4–12 MHz, was adopted for routine ultrasound (US) and SWE inspections. After the lesion was clearly displayed on the two-dimensional (2D) view, the SWE mode was switched to allow dual dynamic observation of 2D images and elastograms. The appropriate size and position of the sampling frame was selected to ensure the lesion area and normal gland tissue were at the same level, with a relatively fixed probe position and strength for more than 3 seconds. When recording images, the region of interest (ROI) needed to be selected for calculation of the elasticity value, and the sampling frame covered the entire lesion as much as possible, particularly the hardest part of the lesion. For each patient, the maximum, average, minimum elasticity, elasticity ratio of the lesion to the peripheral parenchyma, and the presence of "stiff rim sign" were measured and recorded 3 times.

Domain-specific antibodies aiming at the phosphorylated Smad2 and Smad3

Polyclonal anti-phosphorylated Smad2 and anti-phosphorylated Smad3 antibodies (abs) were proposed for the phosphorylation junction region and COOH-terminal region of Smad2 and Smad3 by immunization of rabbits with synthetic peptides (16). The relevant antisera were affinity purified with the phosphopeptides (17).

Immunohistochemical studies and image analysis

After formalin fixation and paraffin embedding, the lesion was cut into 4 m thick sections. We then detected Smad2/3 in the breast lesions by immunohistochemistry (IHC) as

Table 1 The elasticity parameters and Smad2/3 in benignity and malignancy

Parameter	Benign	malignant	t/χ^2	P
Maximum elasticity (kPa)*	58.2±50.5	161.9±79.5	9.276	<0.001
Mean elasticity (kPa)*	35.9±26.4	99.6±51.9	9.433	<0.001
Minimum elasticity(kPa)	15.4±12.9	20.6±19.5	1.434	0.154
Eratio*	2.1±1.7	6.0±4.1	7.625	<0.001
Stiff rim sign*	4 (4.8%)	38 (74.5%)	72.030	<0.001
Smad2/3*	0.104±0.009	0.299±0.011	16.156	<0.001

*, a value of P<0.05 is considered statistically significant. Eratio, the elasticity ratio of the lesions to the peripheral tissue.

described previously (9). The software Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) was used for image analysis. From each slice, 5 areas of interest were taken under the visual field of 400. The integrated optical density (OD) and area of the region were measured, and the average OD (integrated optical density/area) was calculated. The average OD was used to evaluate the expression level of Smad2/3.

Statistical methods

The statistical software SPSS 26.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Measurement data was described as mean ± SD and count data as a percentage (%). Quantitative data was tested by χ^2 test, and qualitative data was tested by independent sample *t*-test. A receiver operating characteristic (ROC) curve was constructed, and the area under curve (AUC) was used to evaluate the elasticity characteristics, expression level of Smad2/3, and their combined differential diagnosis efficiency for mammary malignant and benign diseases. The Z test was adopted to compare the difference of AUCs. Pearson correlation analysis was performed to evaluate the correlation between Smad2/3 expression level and the elastic parameters of breast lesions. A difference of P<0.05 was considered statistically significant.

Results

Pathology

The pathological results of 135 breast lesions revealed that 84 were benign (62.2%) and 51 malignant (37.8%). The benign lesions comprised 40 fibroadenosis, 32 fibroadenomas, 8 inflammations, 3 papillomas, and 1 benign phyllodes tumors. The malignant lesions involved 46 invasive ductal carcinoma, 3 ductal carcinoma in situ (DCIS), and 2 mucinous carcinomas.

Comparison of elasticity between benignity and malignancy

Table 1 shows the maximum, mean, minimum elasticity, elasticity ratio of lesions to peripheral parenchyma of lesions, and detection rate of "stiff rim sign" of benignity and malignancy. Malignant nodules presented significantly higher maximum, mean elasticity, elasticity ratio of lesions to peripheral parenchyma, and detection rate of "stiff rim sign" than benign nodules (P<0.001, *Figure 1A*,*1B*).

Comparison of Smad2/3 expression level between benignity and malignancy

In all the breast lesions, Smad2/3 had an average OD of 0.142±0.105. Among them, the expression level of smad2/3 in malignant breast tissues was significantly higher than that in benign breast tissues (0.299±0.011 *vs.* 0.104±0.009, P<0.001, *Table 1*, *Figure 2A*,*2B*). The AUC of the differential diagnosis of benignity and malignancy by Smad2/3 expression level was 0.957 (0.906–0.985, *Figure 3*). When the mean OD cutoff value was 0.1495, the sensitivity and specificity of differential diagnosis of benignity and malignancy were 94.00% and 91.36%, respectively.

Correlation between elasticity and Smad2/3 expression level

The study showed that the expression level of smad2/3 was positively correlated with the maximum elasticity (r=0.657, *Figure 4A*), the average elasticity (r=0.640, *Figure 4B*), and the ratio of lesions to peripheral tissue (r=0.470, *Figure 4C*). The expression level of Smad2/3 in breast lesions with "stiff rim sign" was significantly higher than that of those without

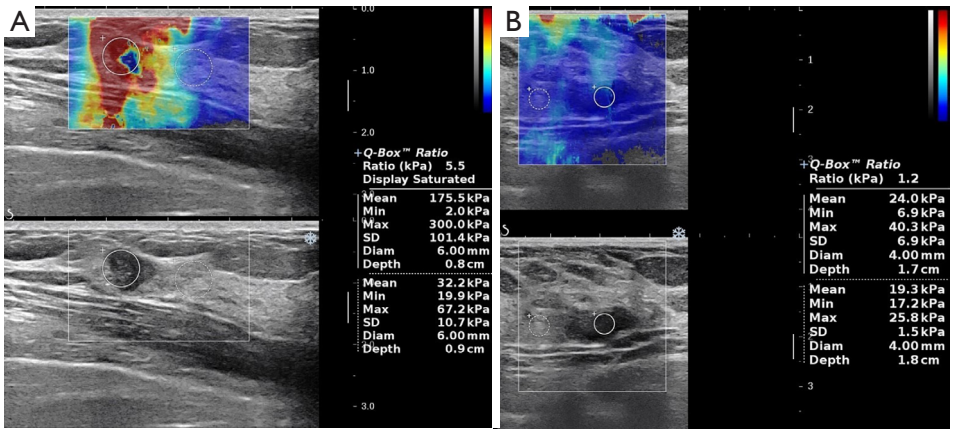

Figure 1 Elastic parameters of breast lesion. (A) SWE image of an invasive ductal carcinoma in a 43-year-old female. The maximum elasticity was 300.0 kPa, the average elasticity was 175.5 kPa, and the elasticity ratio of lesions to peripheral parenchyma was 5.5; (B) SWE image of a fibroadenoma in a 56-year-old female. The maximum elasticity was 49.3 kPa, the average elasticity was 24.0 kPa, and the elasticity ratio of lesions to peripheral parenchyma was 1.2. SWE, shear wave elastography; kPa, kilopascal.

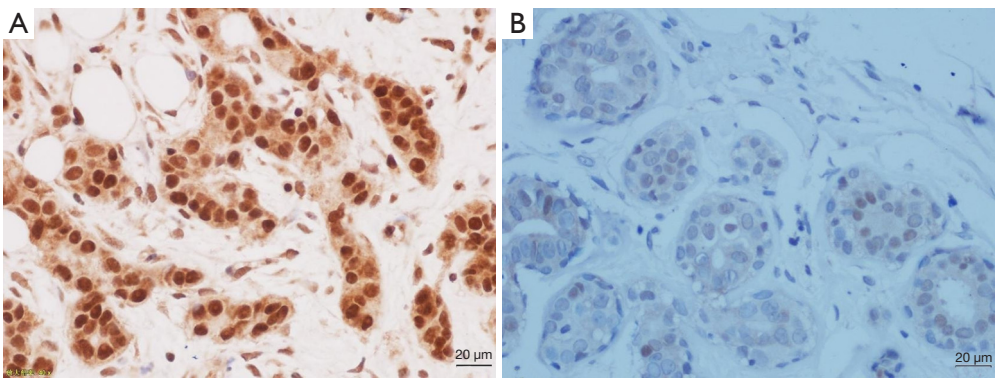

Figure 2 The staining method was immunohistochemical staining with an amplification of 400 times. (A) Smad2/3 expression was strongly positive with an average optical density of 0.2824; (B) Smad2/3 was weakly expressed mainly in cytoplasm and nucleus, with an average optical density of 0.0220.

"stiff rim sign" (0.2512±0.076 *vs.* 0.075±0.050).

Diagnostic properties of Smad2/3 combined with elasticity

Table 2 lists the diagnostic properties of Smad2/3, the maximum, mean elasticity, elasticity ratio of lesions to peripheral parenchyma of lesions, and detection rate of "stiff rim sign" and Smad2/3 combined with "stiff rim sign". Although the Smad2/3 combined with "stiff rim sign" achieved no higher specificity than "stiff rim sign" ($P<0.05$), it yielded the significantly highest diagnostic sensitivity and accuracy in all indexes ($P<0.05$ for all) (*Table 2*).

Discussion

Accumulating evidence has demonstrated that SWE has excellent diagnostic capabilities in distinguishing benign and malignant lump masses, which is conducive to the earlier diagnosis of mammary cancer (18,19). Collagen and fibronectin in ECM, as important factors determining lesion elasticity, play crucial roles in the occurrence and development of breast cancer (14,15). However, the Smad2/3 pathway, as a major pathway controlling the expression of collagen and fibronectin (13), has rarely been studied in relation to the elastic characteristics of breast lesions.

This study indicated that malignant neoplasms exhibit

significantly higher maximum, mean elasticity, and elasticity ratio of lesions to peripheral parenchyma than benign neoplasms, consistent with previous research (19,20). Breast tumors have been reported to develop with ECM stiffening, collagen cross-linking, increased focal adhesions, fibrogenic response, and tumor infiltration into adjacent stroma, which were shown to be responsible for the scirrhous properties of tumors (21-23). Our previous study showed that the diagnostic sensitivity and specificity of US + SWE for breast lesions could reach 88.6% and 90.5%, respectively (20). Also, this research showed that maximum, mean elasticity, elasticity ratio of lesions to peripheral parenchyma, and detection rate of "stiff rim sign" all had high diagnostic efficiency, which further confirmed that SWE was of great value in the differential diagnosis of breast neoplasms.

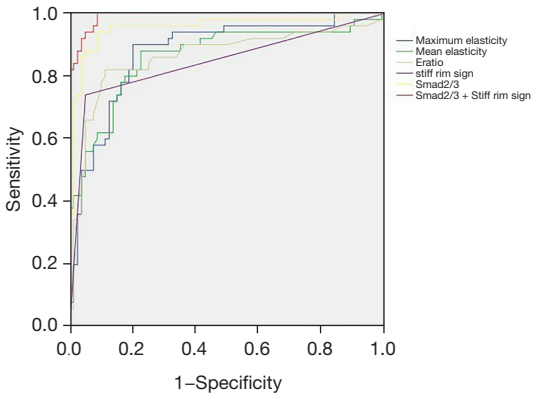

Figure 3 The ROC curve of the differentiating diagnosis of benignity and malignancy by Smad2/3 expression level, the elastic characteristics of lesions, "stiff rim sign" and Smad2/3 combined with "stiff rim sign". Eratio, the elasticity ratio of the lesions to the peripheral tissue.

This study revealed that the expression of Smad2/3 in benign breast nodules was significantly less than that in malignant masses, which was defined as a critical feature for the recognition of malignant masses. Previous studies have gradually revealed that the Smad2/3 pathway could mediate anti-tumor and carcinogenic effects of TGF-β, and that the Smad2/3 pathway mediates the anti-tumor and carcinogenic effects of TGF-β depending on the cell's progression stage and other synergistic contextual changes (6,10). Studies have reported that Smad2/3 is overexpressed in breast cancer (24), pancreatic cancer (25), gastric cancer (26) and other tumors (27,28), which can be used to indicate

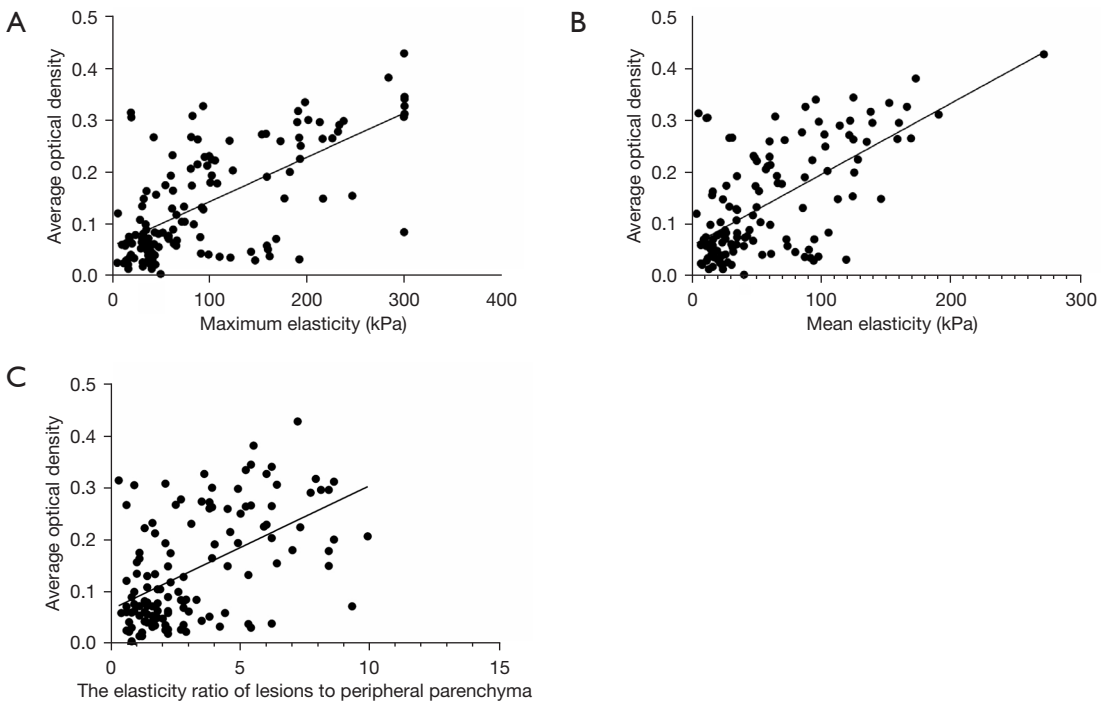

Figure 4 Correlation between Smad2/3 expression level and elastic characteristics of lesions. (A) The correlation between Smad2/3 expression level and maximum elasticity was shown; (B) the correlation between Smad2/3 expression level and mean elasticity was shown; (C) the correlation between Smad2/3 and the ratio of elasticity between lesions and peripheral tissues was shown.

Table 2 Sensitivity, specificity, Youden index and accuracy of the parameters of SWE, the expression level of Smad2/3 and the combination of Smad2/3 and "stiff rim sign"

Parameter	Cutoff value (kPa)	Sensitivity (%)	Specificity (%)	Youden index	Accuracy (%)
Maximum elasticity	>74.8	90.20	79.76	0.6996	83.70
Mean elasticity	>47.9	88.24	77.38	0.6562	81.48
Eratio	>3	82.35	87.95	0.7030	85.83
Stiff rim sign	–	74.51	95.24	0.6975	87.41
Smad2/3	>0.150	94.00	91.36	0.8536	92.36
Smad2/3 combined with stiff rim sign	>0.194	100.00	91.25	0.9125	94.56

Eratio, the elasticity ratio of the lesions to the peripheral tissue; SWE, shear wave elastography.

the prognostic effect of those malignant tumors. A study by Yamagata *et al.* (4) highlighted a significant increase in c-Jun NH2-terminal kinase (JNK)-dependent Smad2/3 as the process of neoplasia progressed from normal colorectal epithelial cells to invasive adenocarcinoma with distant metastasis. Additionally, knocking out the expression of Smad2/3 inhibited the expression of Snail, vimentin, fibronectin, and cancer cell invasion induced by epidermal growth factor (EGF), indicating that the activation of Smad2/3 was associated with EGF-induced EMT in breast cancer (10). Increased expression of Smad2/3 maintains the stem cell characteristics of breast cancer cells, which in turn promotes the proliferation and progression of breast cancer (24). Furthermore, vasculogenic mimicry (VM) formation, via the Smad2/3 pathway, could support growing tumors and promote disease progression *in vitro* and *in vivo* (11). It has been shown that by activating the Smad2/3 pathway, the tumorigenicity and sphere-forming capability of breast cancer cells could be enhanced (29). Thus, in this study, the findings of the higher expression of Smad2/3 in malignant breast lesions than that of benign lesions indicated that Smad2/3 may be partially responsible for the development of breast carcinoma.

This study revealed that the expression of Smad2/3 was related to the maximum, mean elasticity, and elasticity ratio of lesions to peripheral parenchyma of lesions. Literature has demonstrated that the expression of Smad2 and Smad3 is upregulated in cirrhotic livers compared with normal livers (30). In human atherosclerotic lesions, genes activated by TGF-β/Smad signaling that have the potential to affect lesion characteristics include the α1 chain of type I collagen, α2 chain of type I collagen, α1 chain of type III collagen, and connective tissue growth factor (13). A study demonstrated that Smad2 and Smad3 knockdown alleviated the synthesis of collagen I, V, IV, and fibronectin messenger RNA (mRNA) in fibroblast cells both *in vitro* and in adult mice (31). Moreover, it has been shown that Smad2/3 also contributes to inhibiting the degradation of proteins (collagen and fibronectin) in EGM (21,32). Furthermore, via the Smad2/3 pathway, various ECM-remodeling enzymes are induced in breast cancer, which could improve cell adhesion and reduce the activity of the lesion (33). Therefore, this correlation between Smad2/3 and elastic characteristics of breast lesions suggests that Smad2/3 might have a detrimental effect on the formation of tumor stiffness.

In this study, the incidence of "stiff rim sign" in malignant tissues (74.5%) was significantly greater than that in benign tissues (4.8%) (P<0.001), and compared with breast lesions without "stiff rim sign", those with "stiff rim sign" showed higher Smad2/3 expression level, suggesting the expression level of Smad2/3 was also associated with "stiff rim sign". Research has shown that the "stiff rim sign" might stem from the proliferation of connective tissue or the involvement by tumor cells into the peripheral organization, which is more common in malignant breast lesions (20). Also, a previous study showed that fibroblasts and other progenitor cells were converted into myofibroblasts via the Smad2/3 pathway (34). In the highly vascularized desmoplasia of breast cancers, myofibroblast activity shifts outward to the growing edge, producing a 2-fold to more than 10-fold increase in the stiffness of tissues surrounding the tumor (23). Thus, "stiff rim sign" was associated with Smad2/3 expression level, suggesting that Smad2/3 might participate in the formation process of "stiff rim sign".

Previous studies have shown that the "stiff rim sign" held a high diagnostic competence (20,35). In this study, the

specificity of Smad2/3 combined with "stiff rim sign" was lower than that of "stiff rim sign"; however, its diagnostic sensitivity and accuracy was significantly greater than that of "stiff rim sign" (P<0.05 for all). The combination between Smad2/3 and "stiff rim sign" might be a new entry point for the differential diagnosis of breast cancer, which may provide a supplement to the diagnosis of breast lesions.

This study has several limitations. First, the differential role of Smad2 and Smad3 in the process of increasing stiffness of tumor tissue has not been studied. The phenotype of Smad2 and Smad3 knockout mice and the comparison of the activation of Smad2 and Smad3 of TGF-β downstream genes indicated that Smad2 and Smad3 had different functions in TGF-β signal transduction and may play varied roles in the formation of tumor stiffness (6). Second, which regions (N-terminal Mad homology domain-1, intermediate linker, C-terminal Mad homology domain-2) of Smad2/3 had been phosphorylated was not detected. It has been reported that late-stage cancer with deep invasion and/or metastasis is typically characterized by high phosphorylation of Smad3 linker region in colorectal cancer (4), and the relationship between high phosphorylation of distinct roles and the stiffness of breast lesions needs further investigation.

Conclusions

In conclusion, maximum, mean elasticity, elasticity ratio of lesions to peripheral parenchyma, and "stiff rim sign" are correlated with the expression of Smad2/3, suggesting smad2/3 may play a vital role in leading to tumor stiffness. The combination of the expression of Smad2/3 and "stiff rim sign" might contribute to the diagnosis of breast cancer.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The prospective study was approved by the Ethics Committee of the Chinese PLA General Hospital (No. S2020-336-01) and informed consent was taken from all the patients.

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