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Relationship of shear wave elastography anisotropy with tumor stem cells and epithelial-mesenchymal transition in breast cancer

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Abstract
Background: This study is to examine the feasibility of shear wave elastography (SWE) anisotropy in assessing the prognosis of breast cancer.

Methods: We enrolled 119 breast cancer patients from January 2017 to October 2019. SWE was performed before operation. Emax (maximum elasticity value), Emean (average elasticity value), Esd (standard deviation of the lesion elasticity value), Eratio (elasticity value of adipose tissue), anisotropy coefficient and difference were recorded. After operation, we collected clinical pathological data, and performed immunohistochemistry and real-time PCR tests on CD44, CD24, E-cadherin, β-catenin, vimentin and N-cadherin. Finally, we analyzed the correlation among parameters of SWE, anisotropy and clinicopathology, and markers of CSCs (cancer stem cells) and EMT (epithelial-mesenchymal transition).

Results: Emax, Emean and Esd of the cross section were higher than those of the longitudinal section. Breast cancer with a higher elastic modulus was often accompanied by a hyperechoic halo, which was manifested as mixed echo and post-echo attenuation, and was accompanied by a higher BI-RADS (breast imaging reporting and data system) classification. When breast cancer had hyperechoic halo and weakened posterior echo, SWE of the lesion showed more obvious anisotropy. In addition, larger diameter of the longitudinal section indicated higher stiffness of the cross section. Correlation analysis showed that E-cadherin was negatively correlated with SWE in longitudinal section. CD44, N-cadherin, β-catenin were positively correlated with SWE in longitudinal and cross sections. Vimentin and CD24 had no correlation with SWE parameters.

Conclusion: SWE of breast cancer is anisotropic. The cross-sectional SWE is better than the longitudinal SWE, Emax is better than Emean, the anisotropy of SWE is better than SWE, and the anisotropy factor is better than the anisotropy difference.

Keywords: Anisotropy, Breast cancer, Cancer stem cells, Epithelial-mesenchymal transition, Shear wave elastography (SWE)

Background
Studies [1, 2] have shown that tumor stiffness is determined by the stiffness of the matrix. The extracellular matrix is composed of a network of biopolymerized fibers. The density of extracellular matrix is determined...
by the collagen content, fiber thickness, internal fiber cross-linking degree, and the porosity of the extracellular matrix, which further determine the mechanical properties and rheological properties of the extracellular matrix [3]. With the abnormal deposition and cross-linking of the extracellular matrix, as well as the increase in matrix stiffness, the mechanical properties of the tumor microenvironment will change [4], causing the stiffness of tumor tissue [5]. Tumor stiffness can be reflected on ultrasonic images. However, ultrasonic images are affected by uncertainties and/or inaccuracies of various kinds which, among other things, determine an extremely low quality grayscale image contrast. This contrast has been treated by using fuzzy techniques precisely [6, 7]. Among them, shear wave elastography (SWE) is a stable ultrasonic elastic technology with high repeatability and is independent of external pressure, which reflects the stiffness of the tissue to a certain extent [8]. By exciting and precisely controlling the propagation and capture of shear waves in the human body, SWE presents the elastic modulus of the tissue in real time. However, breast cancer is a highly heterogeneous tumor. In clinical practice, we observe that SWE of breast cancer is anisotropic. SWE quantitative parameters can quantify anisotropy and have certain research value. With the occurrence and development of breast cancer, its physical and chemical properties will change [9]. It is found that the anisotropy of breast lesions may be related to the degree of tumor malignancy [10], which is only limited to diagnostic evaluation. In clinic, a method to non-invasively assess the prognosis of breast cancer before surgery is needed.

Although the clinicopathological features of tumor size, pathological type, axillary lymph node involvement and molecular subtypes of breast cancer are all related to the prognosis, studies have shown that cancer stem cells (CSCs) and epithelial-mesenchymal transition (EMT) can better reflect the prognosis of breast cancer than clinicopathological features [11], and they are the key regulators of breast cancer aggressiveness [12]. CSCs of breast cancer have self-renewal and multidirectional differentiation capabilities, which are closely related to tumor occurrence, proliferation, metastasis, and drug resistance [12]. EMT causes epithelial cells to lose tight junctions and polarity, and acquire the characteristics of mesenchymal cells, which is always accompanied by up-regulation of N-cadherin and down-regulation of E-cadherin, and is closely related to tumor invasion, metastasis and treatment resistance [13].

One previous study has quantitatively assessed the relationship between breast cancer ultrasound characteristics and biological characteristics through automated imaging omics methods, which showed that the accuracy of ultrasound in predicting breast cancer hormone receptor expression level was 67.7% [14]. This indicates that tumor features at the genetic and cellular level could be reflected through ultrasound imaging. In this study, through correlation analysis, we aim to use SWE to predict the prognostic characteristics of breast cancer at the cellular and molecular levels. We analyzed the anisotropic quantitative parameters of SWE reflecting tumor stiffness and analyzed their correlation with prognostic factors. We measured the expression of CSCs and EMT markers in breast cancer tissues, as well as their correlations with SWE parameters. Our findings may guide the assessment the clinical prognosis of breast using a non-invasive and convenient imaging method.

Methods
Subjects
We included patients who underwent breast cancer surgery at the Department of Breast Surgery of Xinjiang Cancer Hospital from January 2017 to October 2018 and were confirmed to have breast cancer by pathological examination after the operation. All patients underwent routine ultrasound and SWE examinations before surgery, and clinical pathological data were collected after surgery. The exclusion criteria were: (1) patients had undergone neoadjuvant chemotherapy or radiotherapy; (2) patients had recently received a needle biopsy; (3) patients had a history of prosthesis implantation during pregnancy or lactation; (4) patients had a history of surgery on the ipsilateral breast, and had scar tissue adjacent to the lesion; (5) patients with non-mass lesions (such as simple diffuse calcification) on ultrasound; (6) patients with incomplete histopathological data; (7) patients with lesions that cannot be covered by the SWE sampling frame. Finally, 119 patients with breast cancer were included as the study cohort. The age range of the patients was 25–84 years old, with an average age of 48.75 ± 12.12 years old. Tumor samples were obtained by puncture.

Written informed consent was obtained from every patient and the study was approved by the ethics review board of the Affiliated Tumor Hospital of Xinjiang Medical University (No.: G-202102).

Conventional ultrasound and SWE
SuperSonic Imagine’s Aixplorer (SuperSonic Imagine, Aix-en-Provence, France), which had built-in real-time SWE, and was with L1 5–4 linear array probe and frequency 4–15 MHz, was used. After the patients’ bilateral breast and axilla were fully exposed, we performed continuous radial scans centered on the nipple to observe in detail the tumor size, shape, internal echo, boundaries, borders, intralesional calcification, posterior echo attenuation, and blood flow. First, we acquired the best
conventional ultrasound image, and then switched to SWE mode (default range 0–180 kPa, "STD" mode (standard mode)). When the lesion was larger or the site was deeper, we adjusted the ultrasound to the "PEN" mode (penetration mode) to perform qualitative and quantitative SWE observation of the lesion. Subsequently, the probe was rotated along the center of the lesion to the vertical section (longitudinal section) of the largest diameter section, and the previous operation was repeated. Finally, we accessed three independent SWE images in the horizontal and vertical sections, respectively, with the built-in "Q-Box" quantitative software. The sampling frame included the entire lesion and the adjacent hard areas as much as possible. The maximum elasticity value (Emax), the average elasticity value (Emean) and the standard deviation of the lesion elasticity value (Esd) of the entire lesion were recorded. The ratio of the average elasticity value of the hardest part of the lesion to the elasticity value of adipose tissue (Eratio) was calculated. The anisotropy coefficient for each quantitative parameter, including anisotropic difference (AD) and anisotropy factor (AF), is calculated as follows: \( \text{AF} = \left( \frac{\text{cross-section elasticity value}}{\text{longitudinal-section elasticity value}} \right)^2 \) and \( \text{AD} = \left( \frac{\text{cross-section elasticity value} - \text{longitudinal-section elasticity value}}{\text{cross-section elasticity value} + \text{longitudinal-section elasticity value}} \right)^2 \).

Immunohistochemistry

The tumor tissues were sliced and immunohistochemically stained by Envision two-step method to detect EMT markers, including E-cadherin, β-catenin, vimentin, N-cadherin, and CSCs markers CD44 and CD24. Briefly, after antigen retrieval, the endogenous peroxidase was inactivated with 3% H₂O₂. Then, the primary antibodies of anti-E-Cadherin (ab40772, Abcam), anti-β-catenin (ab32572, Abcam), anti-Vimentin (ab8978, Abcam), anti-N-cadherin (ab76011, Abcam), anti-CD44 (ab157107, Abcam), and anti-CD24 (ab124900, Abcam) were added and incubated at 4 °C overnight. On the second day, the sample was incubated with secondary antibody for 1 h at room temperature. After that, the samples were washed 3 times with PBS and developed for 20 min at room temperature. Five fields were randomly observed, and 100 tumor cells were counted. The staining intensity and the percentage of tumor cells with positive staining were used for scoring. Among them, the staining intensity was divided into four levels: negative (no staining), weakly positive (light yellow), positive (brown yellow), and strongly positive (tan) staining. In addition, the percentage of positive tumor cells was also divided into negative (−), very weak positive (+), weak positive (++), positive (+++), and strong positive (+++) staining.

Real-time PCR

Total RNAs were extracted from breast cancer tissues with TRIzol (Transgene, ET111) and reverse transcribed into cDNA with TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgene, AT311). The real-time PCR was performed with Quant-iT Nava SYBR Green Kit (208,054, Qiagen, USA) and on ABI7500 (ABI, USA). The primer sequences for E-cadherin, β-catenin, vimentin, N-cadherin, CD44, CD24 and actin were shown in Table 1. The reaction system was: 2 × SYBR Green Select Mix (5 μl), Forward Primer (0.7 μl), Reverse Primer (0.7 μl), ROX (0.05 μl), cDNA (1 μl), and RNase-free Water (Up to 10 μl). The reaction conditions were pre-denaturation at 95 °C for 2 min; denaturation at 95 °C for 30 s, annealing/extension at 60 °C for 30 s, 40 cycles. The \( 2^{-\Delta\Delta Ct} \) method was used to calculate the relative expression of each gene.

Statistical analysis

Data analysis was performed using SPSS21.0 software, and \( P < 0.05 \) was considered as statistically significant. All data are expressed as mean ± standard deviation (SD). If the data conforms to the normal distribution, one-way analysis of variance was used for multiple comparisons followed by the Sidak method (uniform variance) or the Tamhane method (non-uniform variance). If the data do not conform to the normal distribution, logarithmic transformation of the data was first performed to normalize the data, and then one-way analysis of variance or

| Genes       | Primers | Sequence (5' to 3') | Product (bp) |
|-------------|---------|---------------------|--------------|
| E-cadherin  | Forward | CGAGAAGTCACGTTCAGGG | 119          |
|             | Reverse | GGGGTGTGAGGAGAGAGAAGG |            |
| β-catenin   | Forward | AGCTCCAGAAGCCTACAT | 98           |
|             | Reverse | CGGTCAAACGGCTGTTTCTAC |          |
| Vimentin    | Forward | AGTCCATCGAGTACCGAGAC | 98           |
|             | Reverse | CATTTCACGACATCGTGGCTTC |         |
| N-cadherin  | Forward | AGCCCAACCTTAACTGAGAGT | 136         |
|             | Reverse | GGCAAGGTGAGGAGAGGAGT |            |
| CD44        | Forward | CTGCCGGTTTGGCAGGTGTA | 109         |
|             | Reverse | CATTGTGGGAGGAGGCTATT |           |
| CD24        | Forward | CTCTACCCACGCCAGATTTC | 166         |
|             | Reverse | AGAGTGAGACCACAGAGAGAC |          |
| hsa actin   | Forward | AACAGAGGCCAGCTTGGGCC | 250         |
|             | Reverse | GAGGATGAGCTCAGCTTTCTG |          |

Table 1  Real-time PCR primer sequences
the Wilcoxon rank sum test was used. Pearson’s correlation analysis was used for correlation analysis.

Results
The SWE of breast cancer is anisotropic
In order to clarify whether there is anisotropy in SWE of breast cancer, we first explored the difference of SWE parameters in longitudinal and cross sections. The results showed that the Emax, Emean and Esd of all the cross-section lesions were significantly higher than the longitudinal section (P < 0.05), in which Emax: (139.87 ± 92.64) kPa vs. (133.28 ± 90.80) kPa, P = 0.001; Emean: (45.22 ± 26.54) kPa vs. (42.65 ± 24.92) kPa, P = 0.001; Esd: (15.971 ± 9.096) kPa vs. (18.806 ± 14.482) kPa, P = 0.0161. However, there was no statistically significant difference in Eratio between the cross section and the longitudinal Sect. (11.40 ± 7.79 vs. 10.96 ± 7.50, P = 0.075). As shown in Fig. 1, the breast cancer lesion had anisotropy of SWE parameters, and the SWE elastic modulus in the cross section was significantly higher than that in the longitudinal section. The above results indicate that breast cancer SWE is anisotropic, and the elastic modulus value of the cross section is higher than that of the longitudinal section.

The SWE and anisotropy parameters of breast cancer have a strong relation with conventional ultrasound signs
In order to clarify whether breast cancers with different ultrasound characteristics have different SWE anisotropies, we then analyzed the differences between SWE parameters, conventional ultrasound features and BI-RADS (breast imaging reporting and data system) classification (Table 2). The results showed that SWE parameters were significantly related with breast cancer boundary, internal echo, posterior echo and BI-RADS. This indicates that breast cancer with higher elastic modulus tends to be accompanied by hyperechoic halo, showing mixed echo and posterior echo attenuation, and has a higher BI-RADS classification. Among them, the relation of Emax and Emean with conventional ultrasound signs was stronger than that of Esd and Eratio. The elastic modulus of the cross and longitudinal sections of breast cancer had obvious relation with the boundary of the lesion, internal echo, and posterior echo. However, the relation between the elastic modulus of the cross section and the BI-RADS classification was better than that of the longitudinal section. In addition, the anisotropy parameters of breast cancer were significantly related to conventional ultrasound signs. When there were hyperechoic halo and attenuated posterior echoes in breast cancer lesion, the SWE of the lesions presented more obvious anisotropy, the blood supply of the lesion was less, and the anisotropy of SWE was more obvious. However, the anisotropy of SWE was not related to internal echo and BI-RADS classification. In addition, the anisotropy parameters of Admax, Admean, Adsd, Afratio were positively correlated with the distance between the mass and skin (Table 3). However, the Young’s modulus values of SWE were not related to the distance of the mass surface and body surface (Table 4). In addition, Admax, Admean, ADratio, Afmax, Afmean had a significant positive correlation with the maximum diameters of the ultrasonic cross section and longitudinal section. Each SWE parameter of the cross and longitudinal sections

![Fig. 1](image-url) The anisotropy of SWE of breast cancer on cross section and longitudinal section on ultrasound. The representative ultrasound images of a patient of 66 years old with triple-negative breast cancer were shown. The size of the lesion was about 1.9 × 1.1 cm, with histological grade 3 and clinical stage 1. The cross section of SWE had a higher elastic modulus value than the longitudinal section, showing anisotropy. A: The elastic modulus parameters Emax, Emean, Esd, Eratio of breast cancer on cross section of breast cancer SWE. B: The elastic modulus parameters Emax, Emean, Esd, Eratio of on the longitudinal section of breast cancer SWE.
| Items          | Grouping                        | Cases | E-maximum cross section diameter (mm) | E-maximum longitudinal section diameter (mm) | Emax (cross section) (kPa) | Emax (longitudinal section) (kPa) | Emean (cross section) (kPa) | Emean (longitudinal section) (kPa) | Esd (cross section) (kPa) | Esd (longitudinal section) (kPa) | Eratio (cross section) | Eratio (longitudinal section) |
|---------------|---------------------------------|-------|--------------------------------------|----------------------------------------------|---------------------------|----------------------------------|---------------------------|----------------------------------|---------------------------|----------------------------------|---------------------------|----------------------------------|
| Boundary      | Closely connected               | 62    | 17.912±4.994                         | 17.321±4.354                                 | 86.060±45.765             | 82.914±41.734                   | 55.481±34.786             | 60.905±31.074                   | 13.460±5.205               | 15.595±6.374                     | 7.350±4.199                | 7.669±3.168                     |
|               | Hyper-echic halo                | 57    | 20.514±6.944                         | 19.631±6.713                                 | 120.400±68.395*           | 117.066±75.434*                | 79.765±43.264*            | 79.949±48.472                   | 18.822±11.519              | 22.451±19.556                    | 10.386±7.429               | 8.578±6.872                     |
| Internal      | Mixed echo                      | 38    | 21.589±5.632                         | 19.822±4.896                                 | 142.328±63.540            | 155.900±82.714                 | 90.800±42.617             | 102.994±55.940                   | 21.889±10.111              | 31.072±24.808                    | 10.678±5.843               | 10.406±8.174                    |
| echo          | Low echo                        | 81    | 18.405±6.071                         | 18.015±5.854                                 | 90.285±53.415**           | 82.105±42.272**                | 59.789±37.496**           | 60.036±29.569                   | 14.225±8.061                | 15.187±6.310                    | 8.210±5.109                | 7.413±3.802                     |
|               | No change                       | 47    | 17.444±6.006                         | 16.759±6.151                                 | 92.926±57.482             | 96.755±70.160                  | 59.650±36.002             | 69.303±43.949                   | 13.921±7.847                | 19.674±19.992                   | 8.535±7.042                | 8.332±6.724                     |
|               | Enhancement                     | 35    | 17.455±5.575                         | 17.214±4.701                                 | 71.495±42.604             | 67.145±23.514                  | 44.218±28.780             | 49.309±21.402                   | 11.682±3.602                | 13.023±2.364                    | 6.427±5.031                | 6.864±3.586                     |
|               | Attenuation                     | 37    | 23.226±4.788**                       | 22.052±4.085**                               | 145.083±54.085**          | 132.539±59.082**               | 99.156±37.776**           | 90.217±42.165                   | 23.104±9.385                | 23.056±9.234                    | 11.365±4.500               | 8.922±3.782                     |
| Blood flow    | None or undetectable            | 21    | 14.425±1.521                         | 14.813±1.968                                 | 132.263±56.249            | 129.200±49.720                 | 79.550±25.219             | 81.863±26.564                   | 17.100±5.284                | 18.513±5.406                    | 9.275±3.176                | 9.463±3.689                     |
|               | Intra-lesion signal             | 74    | 19.292±6.277                         | 18.720±5.822                                 | 98.741±57.638             | 93.220±59.850                  | 65.339±39.775             | 67.433±39.759                   | 15.551±9.197                | 18.552±15.679                   | 9.369±6.597                | 8.287±5.663                     |
|               | Increased diffuse blood flow     | 24    | 21.910±5.323*                        | 19.530±6.023                                 | 98.800±72.969             | 109.460±79.270                 | 65.940±55.564             | 74.780±57.509                   | 17.630±11.226              | 20.590±12.336                   | 4.730±1.572                | 5.830±2.108                     |
| BI-RADS       | 4a                              | 18    | 14.220±6.737                         | 12.340±2.848                                 | 87.480±79.255             | 71.800±10.133                  | 50.520±45.899             | 52.520±30.195                   | 10.840±8.271                | 11.700±8.022                    | 5.920±4.272                | 4.440±0.329                     |
|               | 4b                              | 22    | 13.025±5.662                         | 14.988±5.192                                 | 51.588±10.885             | 69.063±15.450                  | 26.788±41.75              | 44.238±21.487                   | 8.725±1.914                 | 13.863±6.050                    | 2.800±0.949                | 8.163±3.551                     |
|               | 4c, 5                           | 79    | 20.242±5.526**                       | 19.302±5.491**                               | 109.382±59.035**          | 104.592±66.123                 | 72.948±39.889**           | 69.339±39.770                   | 17.238±9.196                | 19.944±15.601                   | 9.712±6.111**               | 8.364±5.502*                    |

Emax = maximum elasticity; Emean = mean elasticity; Esd = stand deviation; Eratio = elasticity ratio; BI-RADS = breast imaging reporting and data system. Compared with corresponding subgroup, *P < 0.05, **P < 0.01
Table 3  Differences of breast cancer SWE anisotropy parameters in conventional ultrasound

| Items            | Grouping                  | N      | Afmax         | Afmean         | Afsd          | Afratio       | Admax (k Pa) | Admean (k Pa) | Adsd (k Pa) | Adratio       |
|------------------|---------------------------|--------|---------------|---------------|---------------|---------------|--------------|--------------|-------------|---------------|---------------|
| Boundary         | Closely connected         | 62     | 1280.601 ± 2044.651 | 690.760 ± 8439.65 | 30560 ± 43.260 | 14.950 ± 23.245 | 3.072 ± 36.075 | −5.298 ± 26.048 | 0.516 ± 5.180 | −0.311 ± 3900 |
|                  | Hyper-echoic halo         | 57     | 4478.101 ± 5671.559* | 1642.758 ± 1925.874 | 291.792 ± 740.536* | 24.607 ± 39.957 | 3.314 ± 670.758  | −0.184 ± 41.090 | −3.630 ± 16.922 | 1.808 ± 4.683* |
| Internal echo    | Mixed echo                | 38     | 4006.894 ± 6037.150 | 1597.243 ± 2070.524 | 490872 ± 1032.938 | 10.607 ± 20.471  | −13.572 ± 63.620 | −12.194 ± 39.163 | −9.183 ± 20.747 | 0.272 ± 3.340  |
|                  | Low echo                  | 81     | 2436.582 ± 3819.525 | 1012.039 ± 1307.210 | 53683 ± 77.4456  | 22.334 ± 34.787 | 8.180 ± 490.083  | −0.248 ± 32.075 | −0.962 ± 7.324  | 0.797 ± 4.697   |
| Posterior echo   | No change                 | 47     | 2243.592 ± 3927.642 | 898.829 ± 1527.459 | 258631 ± 767.832 | 6.780 ± 13.110  | −3.809 ± 48.627  | −965 ± 29.280   | −5.587 ± 15.300 | 0.203 ± 2.673   |
|                  | Enhance                   | 35     | 1289.005 ± 2505.800 | 721.051 ± 937.549  | 21374 ± 26.313   | 34.317 ± 40.921 | 4.350 ± 36.477  | −509 ± 26.986   | −1.341 ± 15.309 | −0.436 ± 5.979   |
|                  | Attenuation               | 37     | 4950.947 ± 5693.742** | 1876.636 ± 1722.928* | 112526 ± 118.505* | 24.394 ± 36.910** | 12.543 ± 70.792 | 8.939 ± 43.340 | 0.048 ± 10.846  | 2.443 ± 4.389   |
| Blood flow       | Not shown                 | 21     | 5089.468 ± 4953.320 | 1533.134 ± 1792.920 | 69486 ± 81.171  | 5.916 ± 6.257  | 3.063 ± 76.196  | −2.313 ± 41.786 | −1.413 ± 8.783  | −0.188 ± 2593   |
|                  | Increased diffuse blood   | 74     | 2868.813 ± 4536.686 | 1244.147 ± 1548.292 | 184157 ± 584.223 | 23.621 ± 35.432 | 5.521 ± 54.161 | −2.093 ± 35.793 | −2.953 ± 13.353 | 1.081 ± 4.818   |
|                  | fluid around the lesion   | 24     | 217.348 ± 315.284** | 108.258 ± 1430.59* | 13678 ± 13.409  | 4.152 ± 5.626  | −10.660 ± 10.735 | −8.840 ± 5.784  | −2.960 ± 2.337  | −1.100 ± 1808   |

Afmax = [(cross-section maximum elasticity value) − (longitudinal-section maximum elasticity value)]^2; Afmean = [(cross-section mean elasticity value) − (longitudinal-section mean elasticity value)]^2; Afsd = [(cross-section stand deviation of elasticity value) − (longitudinal-section stand deviation of elasticity value)]^2; Afratio = [(cross-section elasticity ratio) − (longitudinal-section elasticity ratio)]^2; Admax = cross-section maximum elasticity value−longitudinal-section maximum elasticity value; Admean = cross-section mean elasticity value − longitudinal-section mean elasticity value; Adsd = cross-section stand deviation of elasticity value − longitudinal-section stand deviation of elasticity value; Adratio = cross-section elasticity ratio − longitudinal-section elasticity ratio; BI-RADS = breast imaging reporting and data system. Compared with corresponding subgroup, *P < 0.05, **P < 0.01
was positively correlated with their respective maximum diameter. However, the SWE parameters of the cross section were also positively correlated with the largest diameter of the longitudinal section (Table 4). These results indicate that the larger the lesion, the harder the breast cancer and the more obvious the anisotropy. Moreover, the larger the diameter of the longitudinal section, the higher the stiffness of the cross section.

The SWE and anisotropy parameters of breast cancer are related to clinicopathological features

We further analyzed the difference between cross and longitudinal SWE anisotropy parameters in different clinicopathological characteristics of breast cancer. The results showed that the elastic modulus of breast cancer has no correlation with the patient's age, menopausal status, presence or absence of intraductal cancer, lymph node metastasis and different hormone receptors. However, breast cancers with different clinical stages or different histological grades had significant differences in elastic modulus. Moreover, the higher the clinical stage of breast cancer, the higher the cross-sectional Emax, Emean, and Esd. However, there were no statistically significant differences in Emax, Emean, and Esd of breast cancer in different clinical stages (Table 5). Therefore, breast cancer with higher clinical stage and histological grade also has higher elastic modulus.

Moreover, the anisotropy parameters of SWE of breast cancer were significantly related to histological grade (Fig. 2A and B), indicating that breast cancer with higher histological grade has obvious SWE anisotropy.

Immunohistochemistry and real-time PCR analysis of CSCs and EMT markers in breast cancer

Taking adjacent tissues as a control, immunohistochemical results showed that breast cancer tissues had significantly high expression of CD44 and significantly low expression of CD24 (Fig. 3 and Table 6). In addition, the expression of N-cadherin, β-catenin, and vimentin was high ($p < 0.05$). However, the expression of E-cadherin in cancer and adjacent tissues was not statistically significant ($p > 0.05$, Table 6). Real-time PCR showed that $CD44$, $N$-cadherin, and $β$-catenin mRNAs were highly expressed in cancer tissues, while $CD24$ mRNAs were low in expression compared with adjacent tissues ($P < 0.05$) (Fig. 4 and Table 7). There was no statistically significant difference in the expression of $Vimentin$ and $E$-cadherin mRNAs between cancer and adjacent tissues (Fig. 4 and Table 7). Both the immunohistochemical results and real-time PCR results showed that breast cancer tissues showed the characteristics of CSCs and EMT.

The SWE and anisotropy are related to CSCs and EMT

In order to further clarify the prognostic value of SWE and anisotropy, we conducted correlation analysis on SWE modulus, anisotropy, and markers of CSCs and EMT (Table 8). The results showed that mRNA level of $E$-cadherin was negatively correlated with Emax, Emean, Esd, and Eratio on the longitudinal section,
### Table 5: Differences in SWE parameters of cross and longitudinal section in different clinicopathological characteristics

| Items                  | Grouping            | E-maximum cross section diameter | N   | E-maximum longitudinal section diameter | Emax (cross section) | Emax (longitudinal section) | Emean (cross section) | Emean (longitudinal section) | Esd (cross section) | Esd (longitudinal section) |
|------------------------|---------------------|----------------------------------|-----|----------------------------------------|----------------------|-----------------------------|------------------------|-----------------------------|------------------------|-----------------------------|
| Age (year)             | ≤ 46                | 19.577 ± 5.946                  | 59  | 18.900 ± 0.954                         | 102.180 ± 57.435     | 101.156 ± 62.885            | 68.338 ± 38.690         | 71.626 ± 40.393            | 16.362 ± 9.196          | 19.622 ± 15.969            |
|                        | > 46                | 18.695 ± 6.269                  | 60  | 17.965 ± 0.856                         | 102.108 ± 62.454     | 96.738 ± 61.718             | 65.408 ± 42.831          | 68.068 ± 42.079            | 15.590 ± 9.098           | 18.168 ± 13.044           |
| Menopausal state       | Premenopause        | 19.664 ± 5.602                  | 75  | 18.907 ± 5.804                         | 103.324 ± 57.324     | 68.109 ± 38.616             | 68.584 ± 37.924          | 68.207 ± 36.522            | 16.125 ± 8.773           | 18.025 ± 13.873           |
|                        | Post menopause      | 73.529 ± 50.654                 | 44  | 17.325 ± 5.308                         | 99.438 ± 65.864      | 62.892 ± 46.807             | 62.892 ± 46.807          | 73.529 ± 50.564            | 15.617 ± 9.985           | 20.596 ± 15.957           |
| Clinical stage         | ≤ Stage 2           | 18.859 ± 5.564                  | 64  | 18.391 ± 5.528                         | 88.450 ± 52.468      | 95.564 ± 59.539             | 58.418 ± 35.578          | 67.420 ± 38.675            | 13.959 ± 7.991           | 18.530 ± 14.954           |
|                        | > Stage 3           | 19.471 ± 6.759                  | 55  | 18.471 ± 5.927                         | 119.357 ± 64.277*    | 103.137 ± 65.455            | 77.460 ± 44.254*         | 72.846 ± 44.196            | 18.500 ± 9.680*          | 19.154 ± 14.075           |
| Histological grade     | Level 1             | 17.963 ± 4.995                  | 27  | 16.700 ± 4.955                         | 50.400 ± 17.907      | 56.763 ± 28.027             | 30.338 ± 13.930          | 40.775 ± 25.886            | 10.494 ± 1.756           | 12.281 ± 3.770            |
|                        | Level 2             | 18.000 ± 5.664                  | 45  | 17.689 ± 5.234                         | 96.383 ± 51.845      | 108.626 ± 70.617            | 61.483 ± 31.936          | 74.477 ± 43.752            | 14.114 ± 7.571           | 21.143 ± 19.473           |
|                        | Level 3             | 21.230 ± 6.902                  | 47  | 20.407 ± 6.314                         | 139.881 ± 61.615**   | 112.259 ± 55.712**          | 95.441 ± 42.337**        | 81.470 ± 38.276**          | 21.748 ± 10.643**        | 19.870 ± 9.432**          |
| Intraductal carcinoma  | No                  | 19.390 ± 6.121                  | 70  | 18.798 ± 5.693                         | 101.435 ± 57.772     | 96.686 ± 61.691             | 66.998 ± 39.623          | 68.685 ± 39.943            | 15.585 ± 8.789           | 18.838 ± 15.903           |
|                        | Have                | 18.864 ± 6.123                  | 49  | 18.046 ± 5.658                         | 102.869 ± 62.252     | 101.023 ± 62.922            | 66.708 ± 42.105          | 70.992 ± 42.605            | 16.367 ± 9.499           | 18.774 ± 13.074           |
| Lymph nodes            | Non-metastasis      | 17.159 ± 5.495                  | 72  | 16.906 ± 5.452                         | 96.618 ± 57.523      | 116.771 ± 81.259            | 62.459 ± 35.074          | 80.788 ± 50.560            | 14.435 ± 8.952           | 23.641 ± 22.570           |
|                        | Metastasis          | 19.671 ± 6.173                  | 72  | 18.844 ± 5.702                         | 103.658 ± 60.580     | 94.024 ± 55.318             | 68.060 ± 42.174          | 66.818 ± 37.939            | 16.392 ± 9.161           | 17.481 ± 11.237           |
| Hormone receptor status| ER positive group   | 18.725 ± 5.861                  | 78  | 18.279 ± 5.425                         | 101.821 ± 58.772     | 99.554 ± 61.429             | 65.410 ± 39.100          | 69.748 ± 39.773            | 15.694 ± 8.288           | 18.885 ± 14.704           |
|                        | ER negative group   | 20.059 ± 6.344                  | 41  | 18.900 ± 5.860                         | 106.862 ± 61.826     | 100.000 ± 65.094            | 71.738 ± 43.631          | 70.910 ± 44.605            | 17.107 ± 10.334          | 19.245 ± 14.668           |

**Notes:**
- Esd: Standard deviation.
Table 5 (continued)

| Items                  | Grouping                  | E-maximum cross section diameter | N  | E-maximum longitudinal section diameter | Eratio (cross section) | Eratio (longitudinal section) | Paracancer E-maximum cross section diameter | Paracancer E-maximum longitudinal section diameter | Paracancer Emax (cross section) | Paracancer Emax (longitudinal section) |
|------------------------|---------------------------|----------------------------------|----|----------------------------------------|------------------------|-------------------------------|---------------------------------------------|---------------------------------------------|----------------------------------|----------------------------------------|
| Lymph nodes            | Non-metastasis            | 17.159±5.495                     | 47 | 16.906±5.452                          | 10.865±8.113           | 9.306±7.426                   | 12.724±5.414                              | 15.729±4.995                              | 7.176±3.647                      | 10.159±3.744                          |
|                        | Metastasis                | 19.671±6.173                     | 72 | 18.844±5.702                          | 8.198±5.346            | 7.763±4.460                   | 16.761±7.553                              | 15.163±8.040                              | 9.771±4.597*                     | 9.165±5.733                          |
| Hormone receptor status | ER positive group         | 18.725±5.861                     | 78 | 18.279±5.425                          | 8.867±6.267            | 8.219±5.222                   | 15.585±6.822                              | 15.110±6.156                              | 9.265±4.445                     | 8.865±4.332                          |
|                        | ER negative group         | 20.059±6.344                     | 41 | 18.900±5.860                          | 8.962±5.954            | 8.228±5.335                   | 16.086±7.986                              | 14.941±9.110                              | 9.217±4.794                     | 9.734±6.524                          |

Emax = maximum elasticity; Emean = mean elasticity; Esd = standard deviation; Eratio = elasticity ratio. Compared with corresponding subgroup, *P < 0.05; **P < 0.01
negatively correlated with Eratio on the cross section, and negatively correlated with Afmean. These results indicated that the higher the stiffness of the longitudinal section, the lower expression of E-cadherin in epithelial cells. In addition, β-catenin mRNA was positively correlated with Emax, Emean, and Esd of the cross section, and positively correlated with Admean and Adsd. These results indicated that the higher the stiffness of the cross section, the higher the expression of the epithelial phenotype β-catenin. N-cadherin mRNA was positively correlated with Emax, Emean, and Esd of the longitudinal and cross sections, and was also positively correlated with the maximum diameter of the lesions on the cross and longitudinal sections, indicating that the increases of stiffness of the longitudinal section and the cross section, and the lesion enlargement were always accompanied by the increase of N-cadherin. In addition, Admean and N-cadherin mRNA were positively correlated, indicating that the greater difference in stiffness between the longitudinal section and the cross section, the more obvious the interstitial phenotype. CD44 mRNA was positively correlated with Emax, Emean, Esd, and Eratio of longitudinal and cross sections, and was positively correlated with all anisotropy factors of Amax, Afmean, Afratio, and Afsd, indicating that the greater the difference in stiffness between the longitudinal section and the cross section, the more obvious the phenotype of CSCs. However, the expression of Vimentin and CD24 mRNAs had no correlation with SWE parameters.

Discussion

Anisotropy of SWE

Anisotropy is a term describing directional dependence, which exists in fiber-rich biological tissues. Previous study has confirmed the anisotropy of shear waves in skeletal muscle, myocardium, tendons, and other tissues [15]. Recent study has suggested that the elastic characteristics of normal breast and adipose tissue were also anisotropic [16]. In this study, SWE was used to confirm the anisotropy of breast cancer. The cross section elastic quantitative parameters of breast cancer, such as Emax, Emean, and Esd, were significantly higher than the longitudinal section, suggesting that the stiffness of breast lesions is anisotropic in two perpendicular sections, and the elastic modulus of the cross section is more sensitive than that of the longitudinal section. This may be caused by the shrinkage of tumor cells and the arrangement of collagen fibers at the boundary of the lesion. The tumor invades along the direction of the collagen fiber structure, and then metastasizes, and the largest diameter of the tumor is formed [17], which indirectly explains why the shear wave may spread faster in the growth direction of the tumor. Tumor stiffness is related to the connective tissue proliferative response around the tumor, stromal edema around the tumor, internal tumor fibrosis, calcification, and necrosis [18, 19]. The increase in SWE parameters is caused by compression and reduction of the extracellular space, which is caused by the proliferation of tumor cancer cells and tumor stromal cells [20]. The increase in SWE parameters is also derived...
from the abnormality of the breast cancer nuclei, that is, the shrinkage of the intracellular space caused by the increase and enlargement of the tumor cell nuclei [21]. The stiffness of the tumor increases, and the SWE elastic modulus value of breast cancer also increases, which may lead to more obvious the difference in stiffness in mutually perpendicular sections. This may because the speed of the pulse wave emitted by SWE in the direction perpendicular to the tumor stroma is obviously different from the direction parallel to the tumor stroma.

**SWE anisotropy is reflected in its correlation with conventional ultrasound**

In this study, the SWE parameters of breast cancer with better correlation with conventional ultrasound were

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**Table 6** Immunohistochemical scores of CSCs and EMT in breast cancer tissues (n = 119)

| Groups          | CD24    | CD44    | E-cadherin | N-cadherin | β-catenin | Vimentin |
|-----------------|---------|---------|------------|------------|-----------|----------|
| Cancer tissue   | 1.333 ± 1.047 | 2.931 ± 1.241 | 2.600 ± 1.242 | 2.017 ± 1.235 | 2.586 ± 1.200 | 3.431 ± 0.797 |
| Paracancer tissue | 2.259 ± 1.278 | 1.533 ± 1.060 | 2.948 ± 1.343 | 0.200 ± 0.561 | 1.533 ± 1.125 | 1.933 ± 0.961 |
| \(Z\)           | −2.531  | −3.828  | −1.284     | −4.634     | −2.984    | −4.681   |
| \(P\)           | 0.011   | <0.001  | 0.199      | <0.001     | 0.003     | <0.001   |

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**Fig. 3** Representative immunohistochemical staining results. Magnification: × 200. CD44 was strongly positively expressed in the cell membrane and cytoplasm of cancer cells (+++). CD24 was weakly positively expressed in the cytoplasm of cancer cells (+). E-cadherin was weakly positively expressed in the cell membrane and cytoplasm of cancer cells (+). N-cadherin was strongly positively expressed in the cell membrane of cancer cells (+++). β-catenin was positively expressed in the cell membrane of cancer cells (++). Vimentin was strongly positively expressed in the cytoplasm of the tissue surrounding the cancer nest (+++)
cross section $E_{max}$, longitudinal section $E_{max}$, and cross section $E_{mean}$. The SWE anisotropy parameters of breast cancer that had a good correlation with conventional ultrasound included $A_{max}$, $A_{mean}$, and $A_{sd}$. These results indicate that the SWE value of the cross-section has a higher correlation with the ultrasound characteristics, and $E_{max}$ has the highest diagnostic efficiency among the indicator of SWE. Studies [9, 22] have shown that breast cancer with malignant features on conventional ultrasound usually has a higher stiffness in the cross section, and the stiffness difference between the longitudinal section and the cross section is also greater. This is because the characteristics of the invasive growth of breast cancer, which lead to increased necrosis and repair around the lesion, and result in increased fibrous components, adhesions, and the formation of the surrounding interstitial infiltration zone [23]. The interstitial infiltration zone corresponds to the hyperechoic halo in conventional ultrasound. The dense arrangement of fibrous components in the breast cancer can cause the posterior echo attenuation, and liquefaction and necrosis in the breast cancer can form a mixed echo. These all lead to a decrease in elasticity and an increase in stiffness of the aggressive breast cancer [23]. This study showed that there was no statistical correlation between blood flow and elastic modulus values. However, the blood flow was correlated with anisotropic factors, suggesting that the amount of blood supply does not affect the stiffness of breast cancer, but the SWE anisotropy of breast cancer with poor blood supply is more obvious. This also suggests that a large number of abnormally functioning, disorderly arranged new blood vessels may affect the compactness of the structure of the tumor stroma, thereby affecting the anisotropy [24]. Most of the parameters of SWE in this study had good correlation with BI-RADS, which also confirms that SWE may have a good diagnostic value for breast lesions. However, there was no statistical correlation between the anisotropy parameter and BI-RADS, which indicates that its value in breast cancer diagnosis may be not as good as the elastic

![Fig. 4](image_url)

* $P < 0.05$; ** $P < 0.01$

**Table 7** The relative mRNA level of each gene ($n = 63$)

| Groups        | $E$-cadherin | $\beta$-catenin | Vimentin | $N$-cadherin | CD44       | CD24       |
|---------------|--------------|------------------|----------|--------------|------------|------------|
| Normal        | 1.013±0.179  | 1.015±0.194      | 1.009±0.143 | 1.010±0.155  | 1.011±0.155 | 1.017±0.205 |
| Breast cancer | 0.844±0.304  | 1.178±0.184$^\text{a}$ | 0.948±0.300 | 1.237±0.308$^\text{a}$ | 1.250±0.216$^\text{a}$ | 0.861±0.322$^\text{a}$ |

Compared with normal, $P < 0.05$
Table 8  Correlation of SWE elastic modulus and anisotropy parameters with tumor stem cell and epithelial-mesenchymal transition markers

| Items          | E-maximum cross section diameter | E-maximum longitudinal section diameter | Emax (cross section) | Emax (longitudinal section) | Emean (cross section) | Emean (longitudinal section) | Esd (cross section) | Esd (longitudinal section) | Eratio (cross section) | Eratio (longitudinal section) |
|----------------|----------------------------------|----------------------------------------|----------------------|-----------------------------|-----------------------|-----------------------------|---------------------|-----------------------------|----------------------|-------------------------------|
| E-cadherin     | -0.049                           | -0.089                                 | -0.193               | -0.285*                     | -0.212               | -0.300**                    | -0.186              | -0.247*                     | -0.299**             | -0.232*                       |
| β-catenin      | 0.2                              | 0.141                                  | 0.314**              | 0.008                       | 0.283*                | 0.000                       | 0.288*              | -0.065                      | -0.003               | -0.127                        |
| Vimentin       | 0.091                            | 0.13                                   | -0.148               | -0.057                      | -0.179               | -0.057                      | -0.147              | -0.07                       | -0.221               | -0.164                        |
| N-cadherin     | 0.306**                          | 0.257*                                 | 0.600**              | 0.0415**                    | 0.617**              | 0.419**                     | 0.610**             | 0.257*                      | 0.087                | 0.054                         |
| CD44           | 0.149                            | 0.146                                  | 0.646**              | 0.0560**                    | 0.606**              | 0.532**                     | 0.641**             | 0.509**                     | 0.576**              | 0.553**                       |
| CD24           | 0.016                            | -0.039                                 | -0.063               | -0.178                      | -0.077               | -0.172                      | -0.117              | -0.125                      | 0.04                 | -0.052                        |

| Items          | Admax                            | Admean                                 | ADratio              | Adsd                          | Afmax                  | Afmean                    | Afratio             | Afstd                        |
|----------------|----------------------------------|----------------------------------------|----------------------|-------------------------------|-----------------------|--------------------------|---------------------|-------------------------------|
| E-cadherin     | 0.116                            | 0.108                                  | -0.138               | 0.154                         | -0.179                | -0.223*                  | 0.055               | -0.182                        |
| β-catenin      | 0.343**                          | 0.338**                                | 0.145                | 0.293**                       | 0.001                 | -0.043                   | 0.016               | -0.132                        |
| Vimentin       | -0.1                             | -0.145                                 | -0.11                | -0.028                       | -0.122                | -0.108                   | -0.133              | -0.097                        |
| N-cadherin     | 0.189                            | 0.232*                                 | 0.055                | 0.152                         | 0.095                 | 0.043                    | 0.075               | -0.036                        |
| CD44           | 0.072                            | 0.082                                  | 0.141                | -0.129                       | 0.642**               | 0.602**                  | 0.253*              | 0.0467**                      |
| CD24           | 0.137                            | 0.116                                  | 0.117                | 0.061                         | 0.061                 | 0.047                    | 0.077               | -0.006                        |

*P<0.05; **P<0.01
modulus value. The correlation between the anisotropy parameters of SWE and the measured diameter of the lesions indicates that the anisotropy parameters are more likely to be affected by the size and depth of the mass than the Young’s modulus value. Studies [25, 26] have shown that the larger the lesion, the harder the breast cancer, and the more obvious the anisotropy. However, the larger the diameter of the longitudinal section, the higher the stiffness of the cross section. This is because larger breast cancers often have obvious peripheral connective tissue proliferative responses, interstitial edema around the tumor, internal tumor fibrosis, calcification, and necrosis, making the heterogeneity of the tumor more obvious [27]. The tumor grows along the direction of the collagen fiber structure, that is, the longitudinal section, and the tumor cells on the cross section face the barrier of the collagen fiber structure, which causes the compression and reduction of the extracellular space to form a higher stiffness.

The anisotropy of SWE is reflected in its correlation with clinicopathological manifestations

SWE modulus and anisotropy are determined by breast cancer tumor stroma, and the formation and development of breast cancer tumor stroma affects the morphology, proliferation, migration, invasion and EMT of tumor cells [28]. In this study, we confirmed that the SWE parameters and anisotropy parameters of the cross and longitudinal sections were correlated with some clinicopathological manifestations. When the histological grade is higher, breast cancer has greater stiffness and more obvious SWE anisotropy. The higher the histological grade, the higher the rate of DNA aneuploidy in the nucleus reflecting the growth and differentiation of tumor cells. The tumor’s ability to infiltrate the surrounding structures and the range of infiltration also increase; the proliferative activity and cell density of tumor cells also increase; the tumor cell nucleus and nucleolus also increase; the stiffness of the tissue increases; and, the interstitial fiber framework of the tumor also tends to be perfect, all of which may lead to more obvious anisotropy in SWE [29]. Breast cancer with a higher clinical stage has a higher modulus of elasticity in the cross section, but there is no such correlation in the longitudinal section, which also indicates that the tumor stiffness in the cross section can better reflect the prognosis than the longitudinal section [30, 31]. In this study, there was no difference in SWE between patients with or without ductal carcinoma in situ. The main reason might be due to the complexity of ductal carcinoma in situ. One study has shown that the average Young’s modulus value of ductal carcinoma in situ of medium and high nuclear grade is higher than that of ductal carcinoma in situ of low nuclear grade, which is lower than that of normal glands [32].

SWE anisotropy is related to the markers of CSCs and EMT

Here, we showed that the E-cadherin was negatively correlated with the longitudinal section stiffness, while β-catenin was positively correlated with the cross section stiffness. These results also confirmed that the anisotropy of stiffness was related with the expression of epithelial phenotype makers. The N-cadherin and CD44 had correlations with most SWE parameters, and also showed the best correlation with the anisotropy of different cross-sectional stiffness, indicating that the greater the stiffness of breast cancer, the greater the difference in the stiffness of the cross section. The high expression of CSCs and EMT markers also indicate the worse prognosis of breast cancer. Some study believed that the change in matrix stiffness can trigger the collective migration of cells by promoting the transformation of epithelial cells to mesenchymal cells [33], which also confirms that the increase in tumor stiffness can strengthen the EMT and anisotropy of breast cancer and other tumors. EMT allows differentiated breast epithelial cells to acquire the characteristics of CSCs and breast CSCs often differentiate into cells with different heterogeneities in many ways, and the structure of tumor mesenchyme is also more complicated [34], which makes the heterogeneity of tumor structure more obvious. Different areas and different planes may have different tumor stiffness. The most intuitive imaging manifestation of this stiffness heterogeneity is that there are different Young’s moduli of SWE in different areas. The quantitative parameters of SWE can reflect the tumor heterogeneity of breast cancer stiffness at the same level, and the anisotropy parameters of SWE can also reflect the tumor heterogeneity of breast cancer at different levels. The stiffness and anisotropy of breast cancer tumors can be directly displayed through the macroscopic images of SWE, which indirectly predicts the CSCs and EMT of breast cancer.

Conclusions

In conclusion, this study believes that each parameter of SWE has prognostic value. The cross-sectional SWE parameter is better than the longitudinal SWE parameter. Emax is better than Emean. The anisotropy parameter of SWE is better than the SWE parameter, and the anisotropy factor is better than the anisotropy difference. Our research is helpful to guide the clinical assessment of breast cancer prognosis through non-invasive and convenient imaging.
Abbreviations
SWE: Shear wave elastography; CSCs: Cancer stem cells; EMT: Epithelial-mesenchymal transition; Emax: Maximum elasticity value; Emean: Average elasticity value; Esd: Standard deviation of the lesion elasticity value; Eratio: Elasticity value of adipose tissue: AD: Anisotropic difference; AF: Anisotropy factor; BI-RADS: Breast imaging reporting and data system.

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Authors’ contributions
X.L., F.M. and J.D. conceived and designed the experiments; X.L., R.J. and H.Z. performed the experiments; M.Y. analyzed the data; H.Z. searched the literature; X.L. wrote the paper; F.M. and J.D. reviewed the paper; J.D. collected the fund. All authors read and approved the final manuscript.

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Availability of data and materials
The raw data used and/or analysed during the current study are available from the corresponding author on reasonable request. Dataset deposition is not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All the experiment protocol for involving human data was in accordance with the guidelines of Declaration of Helsinki and the study was approved by the ethics review board of the Affiliated Tumor Hospital of Xinjiang Medical University (No: G-202102). Written informed consent was obtained from every patient.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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