Cytoskeletal and extracellular matrix proteins as markers for metastatic triple negative breast cancer

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Abstract
Objective: This study investigated immunohistochemical staining results of two cytoskeletal proteins (vimentin and cytokeratin-18) and two extracellular matrix proteins (fibronectin-1 and laminin-1 receptor) in different stages of triple negative breast cancer.
Methods: Forty triple negative cancerous breast tissues from patients diagnosed as stage 2A (15), 2B (nine), 3A (10), 3B (four), and 3C (two) were included in this study and were compared with 42 normal breast tissues. Immunohistochemistry results were statistically analyzed using the t-test percent of the StatPac program.
Results: The percentages of positive staining in cancerous tissues for all of the studied parameters were significantly greater than their percentages in normal tissues, except for vimentin. All cancerous tissues from patients diagnosed as stage 3A, 3B, and 3C were positive for both fibronectin-1 and laminin-1 receptor.
Conclusion: Fibronectin-1 and laminin-1 receptor are promising markers for stage 3 triple negative breast cancer.

Keywords
Vimentin, cytokeratin-18, fibronectin-1, laminin-1 receptor, breast cancer tissues, metastasis

Introduction
Triple negative breast cancer (TNBC) is an immunohistochemically defined type of breast tumor. It is classified as basal-like breast cancer and is negative for estrogen

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receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2). TNBC is associated with BRCA1 mutations and is mostly seen in young black and Hispanic women. Incidence-wise, TNBC accounts for approximately 20% of breast cancers in the USA. Clinically, TNBC is characterized by poor prognosis, lack of targeted therapy, and metastasis to the lung, liver, and brain.

Vimentin is a cytoskeletal protein that is a component of class III intermediate filaments. It consists of two polypeptide chains and 466 amino acids. Vimentin is synthesized at high concentrations in non-epithelial cells (mesenchymal cells) and in mammary carcinoma cell lines. Vimentin functions to support the anchoring of organelles within the cytosol and maintains cell shape and cytoplasmic integrity. Vimentin is used as a tumor marker for sarcoma, colon, and upper gastrointestinal tract tumors.

Cytokeratin-18 (CK-18) is a cytoskeletal protein composed of four polypeptide chains. It is highly expressed in liver, placenta, and colon cells and weakly expressed in the lymph nodes of breast carcinoma. CK-18 plays a major role in the liver uptake of thrombin-anti thrombin complexes, interleukin-6-mediated immunity, and the reorganization of filaments. CK-18 is also a classic liver cirrhosis marker.

Fibronectin-1 is a dimer polypeptide with a molecular weight of 220 kDa. It is found in the blood, cell surfaces, and extracellular matrix. It is involved in different vital functions including cell adhesion, blood clotting, immunity, wound healing, and angiogenesis. It has previously been reported that fibronectin-1 is associated with metastasis.

Laminin-1 receptor (40S ribosomal protein SA) is a glycoprotein with one polypeptide chain and a molecular weight of 67 kDa. It is the receptor for laminin-1 glycoprotein. Normally, laminin-1 receptor is expressed on the surface of different cell types. It has been reported that the complex of laminin-1 receptor and its binding protein are overexpressed in some cancers, where it plays a role in tumor metastasis.

This article reports an investigation of the association of positive staining for vimentin, CK-18, fibronectin-1, and laminin-1 receptor with metastatic triple negative breast cancer.

Materials and methods

Study design

This study is an archival, descriptive, and case control study.

Immunohistochemistry

Immunohistochemistry was used to evaluate the expression of vimentin, CK-18, fibronectin-1, and laminin-1 receptor in cancerous and normal breast tissues following typical clinical laboratory procedures. The vimentin and CK-18 kits were purchased from DAKO (Glostrup, Denmark; IS630 and IS618, respectively). Fibronectin-1 staining was done using reagents from Thermo Fisher Scientific (Rockford, IL, USA; Rev 120502) and the laminin-1 receptor was stained following the instructions of US Biological Life Sciences (Marblehead, MA, USA; 030235).

Criteria for immunohistochemistry positive results

A tissue was considered positive if a large number of cells (≥100 cells) was stained positive. Tissues that contained small or medium numbers of positively stained cells were considered negative.

Statistical analysis

The obtained data were analyzed using the t-test percent of the StatPac statistics software. The odds ratios, sensitivity, and specificity were calculated manually.
Odd ratios were calculated using following the equation:

\[
\text{Odds ratio} = \frac{A \times C}{B \times D}
\]

A = the number of cancerous breast tissues positive for the studied parameters  
B = the number of normal breast tissues positive for the studied parameters  
C = the number of cancerous breast tissues negative for the studied parameters  
D = the number of normal breast tissues negative for the studied parameters

Sensitivity is the ratio of the cancerous breast tissues positive for the studied parameters to the total number of positive tissues (cancerous and normal), while specificity is the ratio of normal breast tissues negative for the studied parameters to the number of total negative tissues (cancerous and normal).

**Ethical approval**

An ethical license was obtained from the Sudan Academy of Sciences (SAS). Informed consent was not obtained from the patients because this study was retrospective; however, permission to use the breast tissues was obtained from the Radiation and Isotope Center in Khartoum (RICK) (Sudan).

**Results**

**Study cohort**

The cohort of this study comprised cancerous and normal human breast tissues. Forty triple negative cancerous breast tissues and 42 healthy breast margins were obtained from the archives of the Radiation and Isotope Center in Khartoum (RICK). The breast cancer stage of each tissue sample was retrieved from patient files. Breast cancer stages were routinely determined by imaging techniques such as X-ray, ultrasound, and magnetic resonance imaging.

**Staining results of the normal and triple negative cancerous breast tissues**

The percentages of positive cancerous breast tissues for CK-18, vimentin, laminin-1 receptor, and fibronectin-1 were 85%, 100%, 87.5%, and 87.5%, respectively, while these percentages among the normal tissues were 14.3%, 92.9%, 35.7%, and 33.3%, respectively (Table 1). The percentage of positive cancerous tissues for breast tissues was obtained from the Radiation and Isotope Center in Khartoum (RICK) (Sudan).

| Vimentin | CK-18 | Fibronectin-1 | Laminin-1 receptor |
|----------|-------|---------------|--------------------|
| Control  | Cancerous | Control  | Cancerous | Control  | Cancerous | Control  | Cancerous |
| Negative | 3 0 | 36 6 | 28 5 | 27 5 |
| No (%)   | 7.1% | 85.7% 15% | 66.7% 12.5% | 64.3% 12.5% |
| Positive | 39 40 | 6 34 | 14 35 | 15 35 |
| No (%)   | 92.9% 100% | 14.3% 85% | 33.3% 87.5% | 35.7% 87.5% |
| Total    | 42 40 | 42 40 | 42 40 | 42 40 |
| p-value* | 0.001 | 0.001 | 0.001 | 0.001 |
| p-value** | 0.08 | 0.001 | 0.001 | 0.001 |

*p-value for the comparison between the percentages of positive and negative tissues in one group, i.e., normal or cancerous.

**p-value for the comparison between the percentages of positive tissues in normal and cancerous breast tissues.
CK-18, fibronectin-1, and laminin-1 receptor were significantly higher that their percentages in the normal tissues (p-value \(\leq 0.0001\)). However, vimentin staining showed an insignificant variation between the normal and cancerous breast tissues (Table 1 and Figure 1 and Figure 2).

The odd ratios of breast cancer being positive for the studied parameters

The odd ratios of a Sudanese female with breast cancer were 33.4, 13.9, and 12.3 higher when they were positive for CK-18, fibronectin-1, and laminin-1 receptor, respectively, compared with being negative for these markers (Table 2). The odd ratio of breast cancer positive for vimentin compared with negative for vimentin was not calculated because all cancerous tissues were positive for vimentin (no cancerous breast tissues were negative for vimentin) (Table 2).

Sensitivity and specificity results of positive and negative breast tissues

The sensitivity test showed that a breast tissue positively stained for vimentin, CK-18, fibronectin-1, and laminin-1 receptor had a 60%, 85%, 71%, and 70% chance of being triple negative breast cancer tissue, respectively (Table 3). Moreover, negative staining in breast tissue for vimentin, CK-18, fibronectin-1, and laminin-1 receptor had a 100%, 86%, 85%, and 84% chance of being normal breast cancer tissue, respectively (Table 3).

Staining results in different stages of triple negative breast cancer

There was an insignificant variation between vimentin staining results in normal and cancerous breast tissues (Table 1). Although all the vimentin staining results were positive in all of the studied breast cancer stages, it was
not associated with breast cancer metastasis (Table 4).

CK-18 was positively stained in 66.7% and 50% of stages 2A and 3C cancerous breast tissues, respectively. The difference between CK-18 negative and positive percentages in stages 2A and 3C were insignificant (Table 4).

Fibronectin-1 was positively stained in all cancerous breast tissues of stages 2B, 3A, 3B, and 3C. Fibronectin-1 positive staining can be considered a tissue marker.

Figure 2. Staining results of the extracellular matrix proteins laminin-1 receptor and fibronectin-1 in cancerous and normal breast tissues.

In total, 87.5% of cancerous breast tissues and 35.7% of normal breast tissues were positive for laminin-1 receptor staining. Similarly, 87.5% of cancerous breast tissues and 33.3% of normal breast tissues were positive for fibronectin-1 staining.

Table 2. The odd ratios of the numerators and denominators of the study.

|                | Vimentin | CK-18 | Fibronectin-1 | Laminin-1 receptor |
|----------------|----------|-------|---------------|--------------------|
| Numerator      |          |       |               |                    |
| Cancerous positive | 40       | 34    | 35            | 35                 |
| Normal positive  | 39       | 6     | 14            | 15                 |
| Ratio           | 1.03     | 5.67  | 2.5           | 2.33               |
| Denominator     |          |       |               |                    |
| Cancerous negative | 0        | 6     | 5             | 5                  |
| Normal negative  | 3        | 36    | 28            | 27                 |
| Ratio           | NA       | 0.17  | 0.18          | 0.19               |
| Odd ratio (OR)  | NA       | 33.4  | 13.9          | 12.3               |

The odds of having triple negative breast cancer were 33.4, 13.9, and 12.3 higher if positive for CK-18, fibronectin-1 and laminin-1 receptor, respectively, compared with being negative for the three proteins.
Table 3. Sensitivity and specificity of the studied parameters in triple negative breast cancer.

| Parameter                  | Sensitivity (%) | Specificity (%) |
|----------------------------|-----------------|-----------------|
| Positive cancerous tissues | 60%             | 100%            |
| Total positive tissues     | 85%             | 86%             |
| Vimentin                   |                 |                 |
| CK-18                      |                 |                 |
| Fibronectin-1              |                 |                 |
| Laminin-1 receptor         |                 |                 |
| Negative normal tissues    |                 |                 |
| Total negative tissues     |                 |                 |

The highest sensitivity was for CK-18; CK-18 staining was able to detect 85% of triple negative breast cancers, while fibronectin-1 and laminin receptor-1 staining were able to detect 71% and 70% of triple negative breast cancer cases, respectively. If a breast tissue stained negative for vimentin, there was 100% specificity for normal tissue.

Table 4. Staining results of vimentin, CK-18, fibronectin-1, and laminin-1 receptor in triple negative breast cancer tissues from patients diagnosed as stage 2 and 3.

The vimentin positive and negative tissues among the different stages were significantly different but the results were similar to the staining in normal tissues (Table 1). CK-18 can be a marker for stages 2B, 3A, and 3B. Fibronectin can be a marker for stages 2B, 3A, 3B, and 3C, while laminin can be a marker for stage 3.
for the triple negative breast cancer stages 2B and 3(A, B, and C) (Table 4).

Unlike CK-18 and fibronectin, laminin-1 receptor was positive in 80% and 77.8% of the cancerous breast tissues of stages 2A and stage 2B (p-value = 0.012 and 0.08, respectively). Positive laminin-1 receptor staining may be a tissue marker for stage 3 triple negative breast cancer, as it was positively stained in all cancerous tissues of stage 3(A, B, and C) (Table 4).

Discussion

This study identified that vimentin is expressed in normal and triple negative cancerous breast tissues with insignificant variation; thus, it is not considered a marker for breast cancer metastasis. Positive vimentin staining was associated with a 60% risk of triple negative breast cancer, while negatively stained tissues were associated with 100% of normal breast tissues. Similar to our findings, Heatley et al. concluded that vimentin is not a useful marker for differentiating between benign and malignant breast tumors. However, they found that its expression was significantly correlated with tumor grade.23 Two research groups have stated that vimentin may be a useful tissue marker for the prognosis of breast cancer including triple negative.24,25 This study reported that vimentin cannot be considered a prognostic factor, as it was expressed in all stages of triple negative breast cancer with insignificant variations. Unlike the finding of this study, Vora et al. found that vimentin was expressed in 57% of breast cancer tissues and its expression was an indication of breast cancer aggressiveness.26 Previously, our research group reported that positive vimentin staining was found in all breast cancer tissues compared with 93% of normal breast tissues.27

The results of this study showed that CK-18-positive tissues were significantly more frequent in cancerous than in normal tissues (85% in cancerous tissues versus 14.3% in normal tissues). Positive CK-18 staining was associated with an 85% sensitivity for triple negative breast cancer, while negative CK-18 staining was had an 86% specificity for being normal tissue. However, some previous studies have stated that CK-18 was upregulated in breast cancer tissues, while other studies mentioned that CK-18 was downregulated in breast cancer tissues compared with normal tissues.26-29 In this study, all tissues diagnosed as stages 2B, 3A, and 3B were positive for CK-18, while in stage 3C, some tissues were negative and some were positive with insignificant variation. However, different studies have concluded that aggressive or advanced-stage cancers are associated with loss of cytokeratin.26,28,30

The percentages of positive staining for fibronectin-1 in normal and cancerous breast tissues were significantly different, which means that it can be used as a target for breast cancer therapy or as a tumor marker. Positive tissues for fibronectin-1 were associated with a 71% risk of triple negative cancer, while negatively stained tissues had an 85% specificity for normal breast tissues. Regarding fibronectin-1 in the different stages, it was positive in all tissues from breast cancer patients diagnosed as stage 2A and 3(A, B and C), i.e., it is a good parameter for stage 2B and 3 breast cancer or breast cancer metastasis. Previous studies have shown that fibronectin-1 was positive in 55%, 65.7%, 76.1%, and 81.4% of invasive breast carcinoma.17,31–33 Similar to the finding of this study, many studies have stated that fibronectin-1 is highly associated with breast cancer invasion and metastasis.34–36

Concerning laminin-1 receptor staining, it was positive in 85.7% of the cancerous breast tissues and in 35.7% of normal breast tissues (p-value ≤ 0.0001). The sensitivity of positive laminin-1 receptor staining
was 70% for triple negative breast cancer and the specificity of negatively stained breast tissues for laminin-1 receptor was 84% for normal breast tissue. All of the breast tissues from breast cancer patients diagnosed as stage 3(A, B, and C) were positive for laminin-1 receptor; thus, it is a useful marker for stage 3 disease. Similar to the results of this study, some previous studies have reported a high percentage of laminin-1 receptor positive cancerous breast tissues compared with low percentages in normal tissues.37–39 It has also been reported that laminins and their receptors are involved in breast cancer progression, invasion, and metastasis.16,40–42

As this is a descriptive study, it suffers from all the negative aspects of descriptive studies, such as the small number of samples and the inability to determine causes or correlations. However, descriptive studies are useful precursors for future survey studies.

Conclusions

1. CK-18, fibronectin-1, and laminin receptor-1 may be useful breast cancer markers.
2. CK-18 may be used as a marker for stages 2B, 3A and 3B.
3. Fibronectin-1 could be a promising metastasis marker for stages 2B, 3A, 3B, and 3C.
4. Laminin-1 receptor may be used as a metastasis marker for stage 3 breast cancer.

Author contributions

Mohammed Elimam Ahamed Mohammed designed and supervised the research, performed statistical analyses and wrote the manuscript. Nuha Mohammed Elhassan did the practical work and revised the manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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