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Intracellular Fibronectin Expression in Invasive Breast Carcinoma: Is It Related to Significant Clinicopathological Prognostic Factors?

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Abstract

Background: Fibronectin plays a key role in the extracellular matrix. The expression of fibronectin and its impact on tumor behavior have been studied in several tumors such as breast carcinoma.
Objectives: We aimed at investigating the immunohistochemical expression of intracellular fibronectin in breast carcinoma and its relationship with significant clinicopathological factors.
Methods: This case-control study was conducted on 125 formalin-fixed and paraffin-embedded tissue blocks, including 50 invasive breast carcinomas (tumor group) and their adjacent normal tissue (tumor control group), and 25 normal control samples from mammoplasty (normal control group). The samples were obtained from the pathology archive of Alzahra Hospital, Isfahan, Iran, from 2016 to 2018. All the 125 samples were stained immunohistochemically by the fibronectin antibody. Intracellular fibronectin expression was then compared between the three groups. Moreover, the relationship between fibronectin expression and some clinicopathological factors was evaluated in the tumor group.
Results: Fibronectin staining intensity and extent showed no significant difference between the normal control and tumor control groups (P-Value = 0.65 and 0.065, respectively), while the tumor group showed a significant difference from both normal control and tumor control groups in fibronectin staining intensity (P-Value = 0.002 and < 0.001, respectively) and fibronectin staining extent (P-Value < 0.001). In addition, a significant relationship between fibronectin expression in tumor samples and fibronectin staining intensity and tumor grade was observed (P-Value = 0.01). However, fibronectin expression did not show any significant relationship with age, tumor size, tumor subtype, and lymph node status.
Conclusions: Intracellular fibronectin expression seems to be a tendency observed in some breast carcinomas. Normal breast tissue, whether adjacent to carcinoma or normal control, does not show such a tendency. Despite the significant relationship between fibronectin expression and carcinoma grade, fibronectin expression did not show any significant relationship with tumor size and lymph node status.

Keywords: Breast Cancer, Fibronectin, Immunohistochemistry, Prognostic Factors

1. Background

Cancer is the second leading cause of death throughout the world, and breast cancer is the most common cause of cancer-related death in adult females (1). Official statistics from cancer registry indicate breast cancer as the most common type of cancer among Iranian women (2). Risk factors such as age, geographical variation, age at menarche and menopause, age at first pregnancy, family history, previous benign breast disease, radiation, and lifestyle have been well-documented for breast cancer (3). Yet, there is an increasing list of molecular factors that play some role in the development and/or progression of this type of cancer. The normal components of the extracellular matrix are among these molecules. Fibronectin expression has been observed in various types of malignant tumors. This finding has been associated with invasive behavior and distant metastasis in some studies (4-8).

The extracellular matrix is a regulator of various developmental stages of the breast. It provides structural support for cells, mediates epithelial-stromal communication, and plays a role in cell survival, proliferation, and differentiation. Alterations in the extracellular matrix architecture influence breast tumor progression and metastasis (9). Fibronectin, one of the components of the extracellular matrix, is a heterodimeric adhesive glycoprotein. It can be synthesized as a dimer with two subunits. Three types of domains (i.e., FNI, FNII, and FNIII). are found in

each monomer, which have an affinity for the extracellular matrix proteins and integrin receptors on the cell surface. Fibronectin is found in plasmatic and cellular forms. Plasmatic fibronectin is the soluble form of fibronectin, which is synthesized by hepatocytes and circulates in the blood. Cellular fibronectin is the product of different types of benign and malignant mesenchymal and epithelial cells and deposits as insoluble fibers in the extracellular matrix. In the normal connective tissue framework of organs, fibronectin plays a key role in connecting various components of the extracellular matrix together and connecting matrix to tissue cells. Fibronectin also plays a role in cell growth, proliferation, differentiation, and migration. Some studies have shown modulating the effect of fibronectin in tumors. Altered expression of fibronectin has been found in tumors compared to normal tissues (10-14). Moreover, fibronectin and collagen seem to play a synergetic role in modulating the properties of the tumor extracellular matrix (15).

The extracellular matrix seems to have a regulatory effect on breast cancer (13, 16). The expression of fibronectin in breast cancer has been investigated in some previous studies. Li et al. (17) showed that fibronectin induces an epithelial-mesenchymal transition-like morphological change in MCF-7 breast cancer cells via downregulation of epithelial markers and upregulation of mesenchymal markers. Moreover, they found that fibronectin promotes cell migration and invasion in MCF-7 cells (17). Bae et al. (10) found that fibronectin expression by breast cancer cells was correlated with some clinicopathological factors, including greater tumor size, greater number of involved lymph nodes, tumor histologic type, and high tumor grade. Ioachim et al. (12) found a positive correlation between fibronectin expression and lymph node involvement. Fernandez-Garcia et al. (18) found a relationship between the intracellular expression of fibronectin and distant metastasis.

Expression of fibronectin in breast cancer has also been investigated as a target for anti-tumor treatments in some studies, and some of these studies have achieved promising results in this regard (11, 13, 19-21). So far, several studies have been conducted to examine the issue of fibronectin expression in breast cancer. Most of these studies have examined the extracellular fibronectin, which may be the product of both breast cancer cells and stromal cells of the tumor. To the best of our knowledge, there are only four studies that have examined intracellular fibronectin expression in breast cancer cells (11, 12, 22, 23). Fibronectin present in the microenvironment of breast carcinoma may be the product of both fibroblasts and breast carcinoma cells; this cell product finally deposits as insoluble glycoprotein in the extracellular matrix.

As mentioned previously, most studies focused on the issue of fibronectin in breast cancer have examined fibronectin in the tumor matrix. However, the evaluation of intracellular fibronectin in breast carcinoma cells is a more accurate way to examine the internal characteristics of breast carcinoma cells and the contribution of tumor cells in the composition of the extracellular matrix. Therefore, we conducted this study to investigate intracellular fibronectin expression in invasive breast carcinoma and determine whether or not it is related to significant clinicopathological prognostic factors of this cancer.

2. Objectives

The purpose of this study was to investigate the intracellular expression of fibronectin in breast carcinoma and its relationship with significant clinicopathological factors.

3. Methods

3.1. Study Design and Sample Selection

This case-control study was carried out on 125 formalin-fixed and paraffin-embedded tissue blocks from invasive breast carcinoma (tumor group) and their adjacent normal tissue (tumor control group) and normal control samples from mammoplasty (normal control group). All the tissue blocks were selected based on inclusion and exclusion criteria from the pathology archive of Alzahra Hospital, Isfahan, Iran (a governmental, specialized, and referral hospital with 48 hospital sections and 950 beds), from 2016 to 2018. The inclusion criteria were well fixed breast lumpectomy or mastectomy specimens with the definitive diagnosis of invasive breast carcinoma and complete clinicopathological data having normal tissue adjacent to carcinoma and dissected axillary lymph nodes. Concerning the normal control group, only mammoplasty specimens having normal breast tissue without any kind of breast pathology were included in the study. The exclusion criteria were those carcinoma specimens without adjacent normal breast tissue and/or lacking dissected axillary lymph nodes. Concerning the normal control group, mammoplasty specimens with any kind of breast pathology were excluded from the study. According to these criteria, 12 samples were excluded, and 125 tissue samples were included in the study.

Sample size was calculated using the following formula:

$$n = \frac{(Z_1 + Z_2)^2 \ [p_1 (1 - p_1)] + p_2 (1 - p_2)}{(p_1 - p_2)}$$

Where $Z_1 = 1.96$, $Z_2 = 0.84$, $P_1 = P_2 = 0.5$, expected power (EP) = 80%, and confidence interval (CI) = 95%.

According to this formula, samples were allocated to each group as follows: 50 invasive breast carcinomas (tumor group), 50 normal tissue adjacent to carcinoma (tumor control group), and 25 normal mammoplasty samples (normal control group).

Microscopic slides of the specimens were re-examined by an expert pathologist to confirm the diagnosis prior to performing immunohistochemistry.

3.2. Ethical Considerations

All the samples were formalin-fixed and paraffin-embedded tissue blocks. They were enrolled anonymously in the study. At the time the proposal was approved by the Deputy of Research of Isfahan University of Medical Sciences, ethical code was not allocated to proposals that used tissue blocks as their samples.

3.3. Immunohistochemistry Staining of Fibronectin

All the 125 samples were stained immunohistochemically by fibronectin antibody (mouse antihuman IgG1 monoclonal antibody, DAKO Company, Denmark). Sections were prepared from tissue blocks and immunohistochemical staining was carried out as follows:

Incubation in an oven at 37°C for 48 h, dewaxation by 100% xylol, rehydration by a series of decreasing concentrations of ethanol (100%, 85%, and 75%), rinsing in a 10% phosphate-buffered saline (PBS) solution, incubation in 10% H_2O_2 and methanol for 30 min to prevent endogenous peroxidase activity, rinsing in 10% PBS solution, incubation in a citrate-buffered solution (PH = 6.1) in the microwave for 14 min, rinsing in 10% PBS solution, adding a blocking serum for 30 min to block the endogenous non-specific bindings, drying, adding primary monoclonal antibody, incubation at room temperature for 30 min, rinsing in 10% PBS solution, adding a broad-spectrum secondary antibody for 30 min, adding horseradish peroxidase-streptavidin and diaminobenzidine (DAB) for 30 min and 10 min, respectively, rinsing in 10% PBS solution, dehydration by increasing concentrations of ethanol (75%, 85%, and 100%), and finally, counterstaining with hematoxylin.

Fibroblasts present in the tissue samples were used as a positive internal control for fibronectin. Negative controls were incubated with PBS instead of fibronectin antibody.

The intensity and extent of cytoplasmic fibronectin staining were then examined in epithelial cells of breast carcinoma and normal breast tissue. It should be noted that both epithelial cells and fibroblasts can be stained with fibronectin antibody. However, our study only included the examination of fibronectin expression in epithelial cells. Tumor cells and normal epithelial cells are distinguishable from fibroblasts by their different arrangement, size, and shape. To consider a specimen as positive, at least 10% of the epithelial cells were needed to be stained.

3.4. Fibronectin Immunohistochemical Scoring

Evaluation of fibronectin expression was performed through the determination of the extent (proportion of positive cells) and intensity of immunoreactivity of positive cells.

Intensity was qualitatively evaluated and then translated to four scores as follows (18):

Score 0: negative;

Score 1: mild;

Score 2: moderate;

Score 3: strong.

Staining extent was quantitatively examined as the percentage of stained cells irrespective of staining intensity. It was then classified as follows (18):

0% to 10%;

11% to 25%;

26% to 50%;

51% to 75%;

76% to 100%.

Scoring of the samples was performed using Olympus CX31 dual-head microscope (Japan). We studied the samples at 100× and 400× magnifications for staining extent and intensity, respectively.

The intensity and extent of intracellular fibronectin immunoreactivity were then compared between the three groups. The relationship between intracellular fibronectin expression and some prognostic factors, including age, tumor size, tumor subtype, tumor grade, and lymph node status was also studied in tumor group. Data concerning age, tumor size (greatest tumor diameter), tumor subtype, tumor grade, and lymph node status were all available in the pathology archive of the hospital.

3.5. Statistical Analysis

Data were represented as mean ± standard error of mean (SEM). Fisher's exact test was used to identify any statistical difference between the groups. To analyze the association between fibronectin expression and clinicopathological parameters, the Kruskal-Wallis test or Fisher's exact test was conducted. All the statistical analyses were performed using SPSS, version 23 (SPSS Inc., Chicago, IL, USA). The significance level was considered as P-Value < 0.05.

4. Results

4.1. Clinicopathological Data

The 125 samples studied in this investigation included 50 (40%) tumor samples, 50 (40%) tumor control specimens, and 25 (20%) normal control specimens. The mean age of the normal group was 38.04 years (standard deviation = 6.56). Data concerning age, tumor size, and lymph node status in the tumor group has been presented in Table 1. The frequencies of grade I, grade II, and grade III tumors were 18%, 54%, and 28%, respectively. In addition, 88%, 4%, and 12% of the tumors were invasive ductal carcinoma, invasive lobular carcinoma, and mixed carcinoma, respectively. No missing data was present.

Table 1. Data Concerning Age, Tumor Size, and Lymph Node Status in the Tumor Group

Parameter	Mean ± SD	Median	IQR
Age	45.20 ± 10.18	44	12.25
Tumor size	5.05 ± 2.38	4.50	2.75
Number of involved lymph nodes	3.34 ± 4.09	2	5

4.2. Fibronectin Staining Intensity

Overall, most (76%) samples were negative for fibronectin staining, and 15.2%, 8%, and 0.8% of the samples showed mild, moderate, and strong degrees of staining intensity, respectively. Most stained samples belonged to the tumor group, and most stained samples in this group showed mild staining. The normal control group showed the smallest number of stained samples. Fisher's exact test showed no significant difference between the normal control and tumor control groups in terms of fibronectin staining intensity (P-Value = 0.65), but the tumor group showed a significant difference in terms of fibronectin staining intensity with both normal control (P-Value = 0.002) and tumor control (P-Value < 0.001) groups (Figure 1, Table 2).

4.3. Fibronectin Staining Extent

Overall, most (76%) samples were negative for fibronectin staining (i.e., staining extent of 0 - 10%), and 13.6%, 5.6%, 3.2%, and 1.6% of the stained samples showed staining the extent of 76 - 100%, 11 - 25%, 26 - 50%, and 51 - 75%, respectively. Most stained samples belonged to the tumor group, and most samples in this group showed the staining extent of 76 - 100%. The normal control group showed the smallest number of stained samples. Fisher's exact test showed no significant difference between normal control and tumor control groups in terms of fibronectin staining extent (P-Value = 0.65), but the tumor group showed a significant difference in terms of fibronectin staining extent with both normal control (P-Value = 0.001) and tumor con-

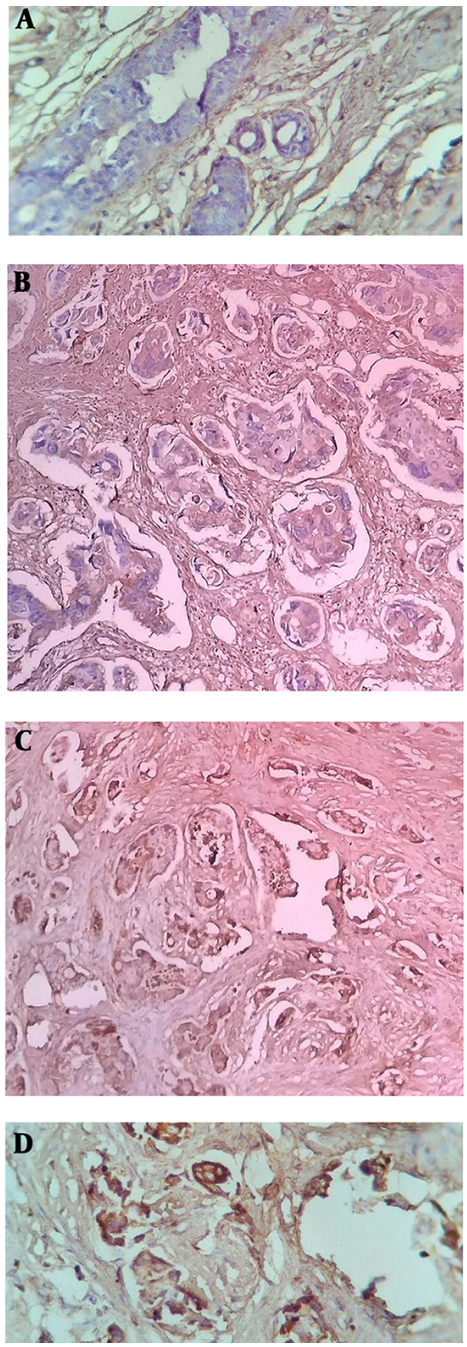

Figure 1. Immunohistochemical staining for fibronectin: A, No staining is seen in the epithelial lining of normal breast duct (40 × objective); B, weak staining is seen in breast carcinoma cells (10 × objective); C, moderate staining is evident in breast carcinoma cells (10 × objective); D, strong staining is evident in breast carcinoma cells (40 × objective).

Table 2. Distribution of Samples in Terms of Fibronectin Staining Intensity and Extent[a]

	Type			
	Normal	Tumor Control	Tumor	Total
Fibronectin staining intensity				
No	24 (19.2)	45 (36.0)	26 (20.8)	95 (76.0)
Mild	1 (0.8)	5 (4.0)	13 (10.4)	19 (15.2)
Moderate	0 (0.0)	0 (0.0)	10 (8.0)	10 (8.0)
Strong	0 (0.0)	0 (0.0)	1 (0.8)	1 (0.8)
Fibronectin staining extent, %				
0 - 10	24 (19.2)	45 (36.0)	26 (20.8)	95 (76)
11 - 25	1 (0.8)	5 (4.0)	1 (0.8)	7 (5.6)
26 - 50	0 (0.0)	0 (0.0)	4 (3.2)	4 (3.2)
51 - 75	0 (0.0)	0 (0.0)	2 (1.6)	2 (1.6)
76 - 100	0 (0.0)	0 (0.0)	17 (13.6)	17 (13.6)

[a]Values are expressed as No. (%).

trol (P-Value < 0.001) groups (Table 2).

4.4. Relationship Between Fibronectin Expression and Prognostic Factors in the Tumor Group

Kruskal-Wallis test showed no significant relationship between fibronectin expression (either intensity or extent of staining) and age (P-Value = 0.92 and 0.86 for intensity and extent of fibronectin staining, respectively).

According to the Kruskal-Wallis test, no significant relationship was found between fibronectin expression (either intensity or extent of staining) and tumor size (P-Value = 0.10 and 0.43 for intensity and extent of fibronectin staining, respectively; Table 3).

The greatest number (70.4%) of tumors was related to invasive ductal carcinoma. No significant relationship was found between fibronectin expression (either intensity or extent of staining) and tumor subtype (P-Value = 0.26 and 0.68 for intensity and extent of fibronectin staining, respectively).

Most tumors (54%) were grade II. The Fisher's exact test showed a significant relationship between fibronectin staining intensity and tumor grade (P-Value = 0.01). However, no significant relationship was found between fibronectin staining extent and tumor grade (P-Value = 0.48; Table 4).

The Kruskal-Wallis test showed no significant relationship between fibronectin expression (either intensity or extent of staining) and the mean number of involved axillary lymph nodes (P-Value = 0.23 and 0.98 for intensity and extent of fibronectin staining, respectively; Table 5).

Table 3. Relationship Between Tumor Size and Fibronectin Staining Intensity and Extent[a]

	Tumor Size	Kruskal-Wallis Test	P Value
Fibronectin staining intensity		4.58	0.10
No	5.13 ± 2.84		
Mild	4.51 ± 1.58		
Moderate	5.75 ± 1.90		
Strong	2.80 ± 0.00		
Total	5.05 ± 2.38		
Fibronectin staining extent		2.71	0.43
0 - 10	5.13 ± 2.84		
11 - 25	7.50 ± 0.00		
26 - 50	4.87 ± 2.28		
51 - 75	6.50 ± 3.53		
76 - 100	4.64 ± 1.48		
Total	5.05 ± 2.38		

[a]Values are expressed as mean ± SD.

5. Discussion

The results of this study showed no significant difference between the normal control and tumor control groups in terms of the intensity and extent of fibronectin staining, while the tumor group showed a significant difference with both tumor control and normal control groups in terms of fibronectin staining intensity and extent. Regarding the relationship between fibronectin

Table 4. Relationship Between Tumor Grade and Fibronectin Staining Intensity and Extent[a]

	Tumor Grade			Total	Fisher's Exact Test	P Value
	I	II	III			
Fibronectin staining intensity					14.89	0.01
No	4 (8.0)	14 (28.0)	8 (16.0)	26 (52.0)		
Mild	3 (6.0)	10 (20.0)	0 (0.0)	13 (26.0)		
Moderate	1 (2.0)	3 (6.0)	6 (12.0)	10 (20.0)		
Strong	1 (2.0)	0 (0.0)	0 (0.0)	1 (2.0)		
Total	9 (18.0)	27 (54.0)	14 (28.0)	50 (100.0)		
Fibronectin staining extent					7.75	0.48
0 - 10	4 (8.0)	14 (28.0)	8 (16.0)	26 (52.0)		
11 - 25	0 (0.0)	0 (0.0)	1 (2.0)	1 (2.0)		
26 - 50	0 (0.0)	2 (4.0)	2 (4.0)	4 (8.0)		
51 - 75	0 (0.0)	2 (4.0)	0 (0.0)	2 (4.0)		
76 - 100	5 (10.0)	9 (18.0)	3 (6.0)	17 (34.0)		
Total	9 (18.0)	27 (54.0)	14 (28.0)	50 (100.0)		

[a]Values are expressed as No. (%).

Table 5. Relationship Between Mean Number of Involved Axillary Lymph Nodes and Fibronectin Staining Intensity and Extent[a]

	Number of Involved Axillary Lymph Nodes	Kruskal-Wallis Test	P Value
Fibronectin staining intensity		2.91	0.23
No	4.57 ± 5.02		
Mild	1.84 ± 2.15		
Moderate	1.70 ± 1.63		
Strong	7.00 ± 0.00		
Total	3.34 ± 4.09		
Fibronectin staining extent		0.18	0.98
0 - 10	4.57 ± 5.02		
11 - 25	1.00 ± 0.00		
26 - 50	1.75 ± 1.70		
51 - 75	2.00 ± 1.41		
76 - 100	2.11 ± 2.42		
Total	3.34 ± 4.09		

[a]Values are expressed as mean ± SD.

expression and clinicopathological prognostic factors in breast carcinoma, a significant relationship was only seen between fibronectin staining intensity and tumor grade.

The established data concerning the presence of a significant relationship between fibronectin expression in breast carcinoma and the known prognostic clinicopathological factors may improve our understanding of breast cancer and have promising impacts on planning further adjuvant treatments for this type of cancer. Thus, we studied cellular fibronectin expression in invasive breast carcinoma, normal breast tissue adjacent to breast carcinoma, and normal breast parenchyma of reduction mammoplasty.

The finding of a significant difference between tumor group and both tumor control and normal control groups in terms of fibronectin staining intensity and extent suggests an alteration of breast cancer cells that results in the high expression of cellular fibronectin in these cells. Since cellular fibronectin is finally released as insoluble fibronectin in the extracellular matrix, its higher concentration in the extracellular matrix of breast cancer in comparison to normal breast tissue would have an impact on growth, proliferation, differentiation, and migration of tumor cells.

A significant relationship between fibronectin staining intensity and tumor grade was seen in the tumor samples. Bae et al. (10) also found a correlation between fibronectin expression by breast cancer cells and high tumor grade. Christensen et al. (22) studied cytoplasmic fibronectin in 24 invasive human breast carcinomas. Sixteen tumors were positive for cytoplasmic fibronectin. The results of their study also showed a positive correlation between the staining intensity of fibronectin and the degree of tumor anaplasia with only a few exceptions. Since

tumor grade is one of the determining prognostic factors in breast cancer, the observed relationship between fibronectin expression and tumor grade may indicate some degree of prognostic significance of fibronectin expression in breast cancer.

According to the results of our study, fibronectin expression was not found to have any significant relationship with age, tumor size, tumor subtype, and lymph node status. However, Bae et al. (10) found that fibronectin expression by breast cancer cells was correlated with greater tumor size, a greater number of involved lymph nodes, and tumor histologic type. Ioachim et al. (12) also found a positive correlation between fibronectin expression and lymph node involvement. Vasaturo et al. (23) evaluated fibronectin by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry in normal breast tissue and benign and malignant breast tumors and correlated it with some clinicopathological parameters. They observed a positive relationship between fibronectin and lymph node status and suggested fibronectin as predictive of long-distance metastasis.

The discrepancy observed between the results of our study and those of Bae et al. and Ioachim et al. (12) studies cannot be attributed to the accuracy of the methods used for demonstrating tissue expression of fibronectin, since immunohistochemistry has been used to investigate fibronectin expression in all of these studies. However, a greater sample size in Bae et al. (10) and Ioachim et al. (12) studies (1596 and 134 samples, respectively) may justify part of this discrepancy. To the best of our knowledge, these four studies (studies of Bae et al. (10), Ioachim et al. (12), Christensen et al. (22), and Vasaturo et al. (23)) are the only studies which have examined intracellular fibronectin expression in breast cancer; other studies have explored the extracellular fibronectin. Our study is only the fifth one that examines intracellular fibronectin expression in breast carcinoma. However, the main limitation of our study was the rather small number of the studied samples due to financial constraints.

5.1. Conclusions

Although a significant relationship was observed between fibronectin expression and carcinoma grade, fibronectin expression was not found to have any significant relationship with two major prognostic factors including tumor size and lymph node status, which have a great impact on tumor stage. The small number of studies concerning the prognostic role of intracellular fibronectin in breast cancer and the presence of some discrepancies in the results of these studies necessitates further studies in this field in the future.

Footnotes

Authors' Contribution: Study concept and design and critical revision of the manuscript for important intellectual content: FM. Analysis and interpretation of data of the manuscript: SH. Drafting of the manuscript: SH. Final approval of the version published: FM.

Clinical Trial Registration Code: This is not a clinical trial study, and we did not require a clinical trial registration code.

Conflict of Interests: The authors declare that they have no competing interests.

Ethical Approval: At the time the proposal was approved by the Vice Chancellery for Research of Isfahan University of Medical Sciences, ethical code was not allocated to proposals which used tissue blocks as their samples.

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