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Original Article

Patients With Invasive Ductal Carcinoma Have Reduced Levels of Decorin Expression in Their Breast Tissue Compared to Patients With Fibroadenoma While Plasma Decorin Remains Unchanged

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Abstract

Background: Reduction in the level of tissue decorin is a hallmark of many types of cancer including breast carcinoma. However, reduced decorin expression has also been reported in several types of benign tumors to the extent that it has been proposed as a tissue marker to differentiate malignant from benign tumors. The aim of this study was to investigate the potential role of plasma decorin to distinguish breast cancer from fibroadenoma, the second most common type of benign tumor, after fibrocystic disease.

Methods: From 35 patients recruited in this study, 24 were affected with invasive ductal carcinoma, either grade II (n = 14) or grade III (n = 10). The other 11 patients had fibroadenoma lesions in their breasts. Tissue decorin mRNA and protein levels were assessed with real-time qPCR and Immunohistochemical analysis. ELISA was employed to measure plasma levels of decorin.

Results: The mean plasma decorin in cancer patients was measured to be 5.42 ± 1.83 ng/mL while fibroadenoma patients had an average of 4.22 ± 1.17 ng/mL decorin in their plasma. The difference was not significant. However, the mean expression level of decorin mRNA calculated by the $2^{-\Delta\Delta Ct}$ method was 5.6-fold lower in the biopsied tissue specimens of IDC patients versus fibroadenoma, as expected. Consistent reduction in protein abundance was observed in the studied tissue sections.

Conclusion: We have shown that tissue decorin is a reliable marker, unaffected by patient disease stage, to differentiate IDC from fibroadenoma. However, plasma decorin does not seem to have diagnostic value in this regard.

Keywords: Breast cancer, ELISA, Fibroadenoma, Invasive ductal carcinoma, Plasma decorin

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Introduction

Despite intensive research and advancement in understanding molecular interactions in tumor microenvironment, breast cancer (OMIM accession number: 114480) diagnosis still, similar to many other types of cancer, relies on histological analysis of tissue biopsies as the gold standard tool to differentiate benign from malignant tumors.[1] Predictions estimated an increased rate of 1.7 million per year to the current annual diagnosed cases of breast cancer and an increase death rate of 0.5 million per year, up to 2025, of which more than 50% belongs to developing countries.[2] However, the actual number of suspected individuals with whom the health care systems are dealing to characterize the tumor type is much more. Fibroadenomas (OMIM accession number: 61554) are one of those suspicious tumor types. As the second most common type of benign tumor after fibrocystic disease,[3] fibroadenoma often appears in young women below the age of 35 years with the overall incidence is2.2% . In spite of the low incidence, 44% to 94% of biopsied breast lesions that undergo pathological analysis in order to evaluate for possible malignancy are eventually diagnosed as fibroadenomas.[4]

Although the promising advancements in imaging techniques such as the combination of dynamic contrast-

enhanced magnetic resonance imaging (DCE-MRI) and dynamic susceptibility contrast magnetic resonance imaging (DSC-MRI)[5] and IR-microspectroscopy,[6] pathological findings are the unique gold standard approach to distinguish fibroadenoma from breast cancer. Therefore, discovering a biomarker that is less invasively accessible is highly attractive. Structurally, fibroadenoma is composed of biphasic proliferation of both stromal and epithelial components and displays a hypo-vascular stroma with intact basement membrane compared to malignant neoplasms.[7] In 0.1%–0.3% of the cases, fibroadenoma may transform to carcinoma.[8] Unlike the fibroadenoma which results from mesenchymal metaplasia and stromal proliferation around the ducts,[9] malignant proliferation of epithelial cells lining the ducts or lobules are breast cancer's major pathological hallmark.[10]

Diverse cellular functions such as proliferation and differentiation in all tissues are closely regulated through the mutual cellular interactions with their microenvironment.[11] Studies concentrated on tumor microenvironment in cancer research illustrated how cancer cells induce their own microenvironment via interaction with the surrounding non-malignant stromal cells and different extracellular matrix (ECM) macromolecules, known to influence the tumor behavior.[12] Of those macromolecules which are abundant in ECM are proteoglycans (PGs) such as versican, lumican and decorin that maintain the structural and functional integrity of the ECM.[13,14] Similar to other PGs, decorin has complex biological activity. It plays a key role in cell signaling and proliferation, differentiation, survival and migration that affect the biology of different types of cancer.[15]

Decorin is synthesized by fibroblasts and myofibroblasts and receives a complex pattern of chondroitin sulfate or dermatan sulfate glycosaminoglycan (GAG) side chain on its core protein before being secreted into the ECM.[15] Several studies have shown that decorin expression is largely reduced or even suppressed by cancer cells, not only in breast carcinoma, but also in a variety of epithelial cancers such as lung, colon and oral cancer and myeloma.[16-20] Therefore, decorin has been proposed as a potential diagnostic[18] or prognostic biomarker, either alone,[21,22] or in combination with the other established primary markers.[23]

Interestingly, reduced decorin expression does not seem to be restricted to malignant tumors but, to some extent, this holds true for several types of benign tumors. Therefore, it has been proposed as a tissue marker to differentiate malignant from benign tumors. For instance, connective tissue stroma surrounding the tumor mass in angiosarcoma and Kaposi's sarcoma is reported to lack decorin expression while comparative legions of hemangioma, a benign type of vascular tumor, represent significant but low expression of decorin.[24] Similarly, liposarcoma and malignant peripheral nerve sheath tumors show lower decorin expression than benign lipoma and neurofibroma.[25] Significant reduction in plasma levels of decorin has also been reported in esophageal squamous cell carcinoma.[26] This phenomenon is of special interest as a non-invasive early diagnostic marker. Recently, the diagnostic value of plasma decorin along with two other

PGs, biglycan and lumican has been reported for urothelial carcinoma of bladder.[14] In the current study, we hypothesized that similar reduction could occur as a consequence of breast cancer and evaluated whether such a reduction could be used to potentially distinguish breast cancer from fibroadenoma.

Patients and Methods
Study Subjects
Thirty-five patients who underwent breast surgery between December 2014 and February 2015 in Mehrad Hospital (Tehran, Iran) or Bahman Hospital (Zanjan, Iran) were recruited in the study. During the surgery, part of the breast biopsies were transferred to RNA stabilization solution-containing tubes (RNAlater; Thermo Fischer Scientific, Waltham, MA, USA) and immediately frozen in liquid nitrogen. The other part was placed in 10% buffered formalin solution for histological analysis. Among the patients, 24 were diagnosed as affected by invasive ductal carcinoma (IDC) based on the pathological records, i.e., analyzing sections obtained from formalin-fixed paraffin embedded tissues. Simultaneous analysis of the other 11 patients' biopsies marked them as fibroadenoma. Clinical stage of the tumors was determined based on the TNM system (tumor size, node, and metastasis).[27] Information on tumor size, clinical stage, histological grade, estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2) amplification status, Ki67, PNI, (LVI, necrosis tumor, micro calcification were obtained from 24 breast cancer patients and are summarized in Table 1.

Ten milliliters blood was withdrawn into EDTA-containing Vacutainer tubes (BD, Plymouth, UK) from every subject. Immediately after collection, samples were centrifuged at 4000 g for 10 minutes and plasma was carefully aliquoted and stored at -80°C until processing. Blood collection from the patients was performed prior to surgery and before starting any treatment. Informed consent was taken from all of the individuals.

Enzyme-Linked Immunosorbent Assay
Decorin levels in plasma of all the subjects were measured using DuoSet enzyme-linked immunosorbent assay (ELISA) kit (DY143 R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. In brief, NUNC ELISA microplates (M9410-ICS, Sigma, St. Louis, MO, USA) were coated with 2μg/mL of Mouse Anti-Human Decorin Capture Antibody (Cat. No: 842254, R&D, Minneapolis, MN, USA) in phosphate buffer saline (PBS) and incubated overnight. A solution of 1% of bovine serum albumin (BSA, A2153 Sigma, St. Louis, MO, USA) in PBS was used to block the plate before incubating an appropriate dilution of plasma samples along with the standard dilution series made in the 1% BSA/PBS for 2 hours. After proper washing, 100 μL of 250 ng/mL biotinylated mouse anti-human decorin (Cat. No: 842225. R&D, Minneapolis, MN, USA) in 1% BSA/PBS was added to each well and incubated for another 2 hours before addition of streptavidin-HRP (Cat. No: 890803, R&D, Minneapolis, MN, USA) (1/200) for 20 minutes. Ultimately, color was developed with the

Table 1. Comparison Between Plasma Protein and Tissue mRNA Levels of Decorin by Clinical and Pathological Characteristics in Patients With IDC

Clinical Variable	Case n = 24 (%)	Plasma DCN (Mean ± SD)	P Value	DCN Relative mRNA Expression (Mean ± SEM)	P Value
ER					
Positive	17 (85)	5.47 ± 2.00	0.48	1.06 ± 0.54	0.25
Negative	3 (15)	4.61 ± 1.28		0.17 ± 0.08	
PR					
Positive	12 (60)	5.68 ± 2.17	0.34	1.36 ± 0.76	0.62
Negative	8 (40)	4.83 ± 1.41		0.27 ± 0.06	
HER2					
Positive	8 (50)	4.83 ± 1.40	0.11	1.84 ± 1.12	0.57
Negative	8 (50)	6.39 ± 2.24		0.2 ± 0.06	
Stage					
IIA	8	5.70±1.79		0.35±0.34	
II B	11		0.15		0.40
IIIA	2	4.37±1.72		2.81±3.82	
IIIC	3				
Grade					
II	14 (58.3)	5.79 ± 1.99	0.25	0.90 ± 0.60	0.34
III	10 (41.7)	4.92 ± 1.53		0.81 ± 0.45	
LVI					
Positive	15 (71.4)	5.68 ± 1.61	0.78	1.25 ± 0.60	0.20
Negative	6 (28.6)	5.04 ± 2.65		0.28 ± 0.14	
PNI					
Positive	5 (23.8)	5.21 ± 2.84	0.93	0.33 ± 0.15	0.59
Negative	16 (76.2)	5.56 ± 1.62		1.18 ± 0.57	
Ki67					
Positive	13 (68.4)	4.54 ± 1.51	0.01	0.66 ± 0.35	0.96
Negative	6 (31.6)	6.75 ± 1.88		1.62 ± 1.39	
Micro calcification					
Positive	15 (68.2)	5.58 ± 1.91	0.03	1.24 ± 0.61	0.39
Negative	7 (31.8)	4.10 ± 1.03		0.28 ± 0.07	
Tumor necrosis					
Positive	11 (52.4)	4.48 ± 1.07	0.04	1.52±0.82	0.51
Negative	10 (47.6)	6.14 ± 2.26		0.37±0.11	
LN status					
Positive	13 (56.5)	5.79 ± 2.03	0.19	1.26 ± 0.71	0.68
Negative	10 (43.5)	5.49 ± 1.54		0.42 ± 0.09	
Tumor size (cm)					
≤2	3 (13.6)	6.27 ± 3.54	0.34	0.11 ± 0.06	0.06
>2	19 (86.4)	5.37 ± 1.60		1.07 ± 0.48	
Menopausal status					
Pre-	12 (50)	4.91 ± 1.40	0.17	2.77 ± 0.96	0.19
Post-	12 (50)	5.93 ± 2.10		0.90 ± 0.70	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor rec2; LVI, lymph vascular invasion; PNI, perineural invasion; Ki67, also known as MKI67; LN, lymph node.
Clinical and pathological characteristics of 24 patients with breast cancer (IDC) are presented. Mean ± SD of plasma protein level of decorin as well as mean ± SEM for relative mRNA expression levels of decorin in corresponding patients are calculated using Independent t-test and Mann-Whitney test. P values < 0.05 are considered as significant.

use of TMB) solution (Cat. No: 555214; Thermo Fisher Scientific, Waltham, MA, USA). We stopped the reaction with 50 µL of (2N H2S04, Merck, Lindenplatz, Germany) per well and measured the optical density on an ELX808 (BioTek, Swindon, UK) microplate reader at 450 and 570 nm. The ODs) that were positioned only within the linear part of the generated four-parameter logistic-log function are considered. Decorin level in every plasma sample was measured at least twice and the values are presented as mean ± standard deviation (SD) calculated from the generated reference curve ranging from 32.1 to 2000 pg/mL. All the steps were performed at room temperature (RT) unless otherwise specified.

RNA Extraction and Real-Time qPCR
Total RNA was extracted from 50–100 mg tissues using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and immediately frozen at -80°C for long term storage. Following DNase I treatment, cDNA was synthesized from total RNA samples using PrimeScript™ RT reagent kit (Takara Bio Inc, Shiga, Japan). 20 ng of each cDNA sample was then used to semi-quantify the

DCN gene (OMIM accession number: 125255) expression in Real-Time quantitative PCR (qPCR) on an ABI StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA,USA) with a SYBRgreen-based method. Table 2 represents primer sequences, their targeting position and expected amplicon sizes. *DCN* transcript levels were normalized against the geometric mean of three reference genes, i.e. glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)* (OMIM accession number: 138400), pumilio-binding family member 1 *(PUM1)* (OMIM accession number: 607204) and ribosomal protein L13-a *(RPL13A)* according to the method described by Vandesompele and colleges.[28-30] Primer sequences are expressed in Table 2. Relative gene expression (fold increase) was calculated by comparison to a single calibrator for all the amplicons with the use of the $2^{-\Delta\Delta Ct}$ method (amplification efficiency was taken into account). Signal intensity generated from a cDNA synthesized from a pool of total RNAs extracted out of five prostate tissue biopsies was taken as calibrator.

Immunohistochemistry

Four-micrometer thick sections were generated from paraffin-embedded breast tissue biopsies. The sections were then deparaffinized with xylene and rehydrated by passing through series of decreasing alcohol concentrations (100%, 90% and 70%). Antigen retrieval was performed in a microwave oven at 900 watt for 5 minutes following another 5 minutes at 180 watt keeping the samples in a commercial solution at pH 6.0 (S1699, DAKO, Glostrup, Denmark). After 10 minutes incubation at RT, slides were treated with a solution of 3% hydrogen peroxide in methanol for 7 minutes and subsequently washed in distilled water and PBS for minutes (5 minutes, each). To stain decorin, a solution of 8μg/ml mouse anti-human decorin monoclonal antibody (MAB143, R&D, Minneapolis, MN, USA) in Antibody Diluent (S0809, DAKO, Glostrup, Denmark) was used in a room-temperatured dark and humid environment. After subsequent washing with IHC wash buffer (1x), PH 7.4 (Dacell, Tehran, Iran) and PBS (5 minutes, each), slides were directly incubated with a solution of peroxidase conjugated goat anti-mouse (K5007; Dako REAL™ EnVision™/HRP, Rabbit/Mouse, Glostrup, Denmark) at RT for 1 hour in a dark environment. Color development was carried out with the use of 0.02% DAB solution (3-3' diaminobenzidine tetrahydrochloride, DAKO, Glostrup, Denmark) for 5 min at room temperature. Counterstaining with modified Harris's hematoxylin was also performed. The intensity of decorin signal was evaluated using the ImageJ software (version 1.43u, NIH, USA) according to the method described by Augoff et al.[31] Stained samples were observed using a light microscope (LX400, Labomed Inc., Los Angeles, USA) and then with a digital camera.

Statistical Analysis

To evaluate distribution of variables, we have used the Kolmogorov-Smirnov test. Values are either expressed as mean ± standard deviation or as percentage numbers when appropriate independent *t* test was performed when values were normally distributed. Otherwise, Mann-Whitney test was applied. For categorical variables chi-square test was used and Pearson correlation coefficients were calculated to evaluate the correlation between decorin and other variables. Logistic regression models were used to determine the independent predictor of breast cancer. *P* values <0.05 are considered as significant. All analyses were performed on SPSS 16.0 (SPSS Inc, Chicago, IL, USA).

Results

Thirty-five patients with breast tumors were recruited in this study. Tumors of 11 patients were diagnosed as fibroadenoma based on the pathological analysis. Of the other 24 patients, 14 tumors were identified as grade II and 10 tumors were identified as grade III IDC tumors. Mean age and menopausal status of all the individuals recruited in the study are presented in Table 3.

Decorin Plasma Protein Levels Do not Differ in Patients With Breast IDC Versus Fibroadenoma

In order to evaluate the applicability of plasma decorin as a biomarker for discrimination of patients with IDC

Table 2. Primer Sequences to Amplify Decorin and Internal Control Genes

Gene	Acc. No./ Gene ID	Sequence 5´ to 3´	Amplicon Size (bp)
GAPDH[29]	NM_00128974502	F: TGGTATCGTGGAAGGACTCATGAC R: ATGCCAGTGAGCTTCCCGTTCAGC	189
PUM1[30]	NM_012423	F: AGTGGGGGACTAGGCGTTAG R: GTTTTCATCACTGTCTGCATCC	100
RPL13A[30]	NM_001020658	F: GAGGCCCCTACCACTTCC R: AACACCTTGAGACGGTCCAG	110

Table 3. Comparison of Age and Menopausal Status of Individuals, Recruited in the Study

Variables	Breast IDC (n = 24)	Fibroadenoma (n = 11)	P Value
Age (mean ± SD)	52.04 ± 13.30	32.55 ± 10.70	<0.0001*
Menopause			
Pre-menopausal, No. (%)	11 (45)	11(100)	0.002 #
Post-menopausal, No. (%)	13 (55)	0	

Age variables are expressed as mean ± SD and menopausal status as percentage numbers in each study group. P values < 0.05 are considered statistically significant and calculated with *independent t test and ⌗chi-square test.

from fibroadenoma, ELISA was employed. As shown in Figure 1, the mean plasma decorin in cancer patients was measured to be 5.42 ± 1.83 ng/mL (n = 24) while the eleven fibroadenoma patients had an average of 4.22 ± 1.17 ng/mL decorin in their plasma. The difference was not significant (*P* = 0.055).

The crude and age-adjusted ORs for the association between fibroadenoma and plasma decorin were 1.67 (95% CI, 0.96-2.88, *P* = 0.06) and 1.06 (95% CI, 0.53–2.12, *P* = 0.86), respectively. The correlation between plasma decorin and age was positive and significant in both IDC and fibroadenoma patients (Pearson correlation: r = 0.41, *P* = 0.04 and r = 0.63, *P* = 0.03, respectively).

Decorin mRNA Expression Is Significantly Decreased in IDC in Comparison With Fibroadenoma

In order to evaluate tissue expression of decorin at the level of mRNA in the studied patients, qPCR was employed. Through normalizing decorin CT values to a combination of three endogenous control genes including *GAPDH*,[29] *RPL13A*,[30] and *PUM1*,[30] which had previously been reported reliable, relative decorin expression was calculated by $2^{-\Delta\Delta Ct}$.
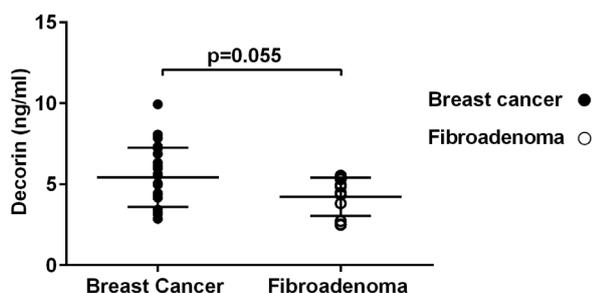
Figure 1. Decorin Plasma Levels in Subjects With Breast IDC Versus Fibroadenoma. Decorin was measured in the plasma of 24 cancer and 11 fibroadenoma patients. The mean ± SD for plasma decorin in study groups were 5.42 ± 1.83 ng/mL (cancer patients) and 4.22 ± 1.17 ng/mL (fibroadenoma). The difference was not significant (*P* = 0.055).
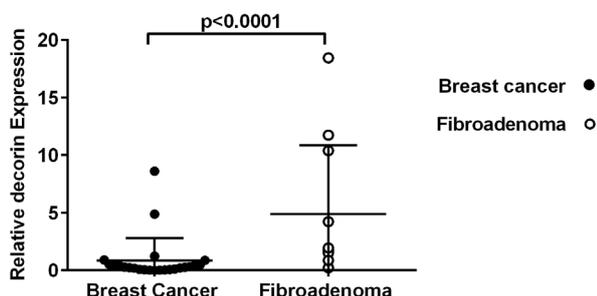
Figure 2. Relative Decorin mRNA Expression in Tumor Biopsies From Breast IDC Versus Fibroadenoma. Biopsies obtained from twenty-four breast cancer and eleven fibroadenoma patients. mRNA expression levels were studied by Real-Time qPCR. Each plot represents decorin expression level in one sample after being normalized to the mean expression levels of *GAPDH, PUM1* and *RPL13-A* genes in that sample. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative decorin expression. Since values were not normally distributed, Mann–Whitney test was applied to calculate the mean and standard errors. The mean of decorin relative expression in breast cancer patients is significantly lower than that in fibroadenoma patients (*P* < 0.0001)

method in tissue extracts from 24 breast adenocarcinoma and 11 fibroadenoma biopsies. Calculated expression levels of each sample are plotted on a scatterplot diagram in Figure 2. As expected, the mean expression level of decorin was lower (5.6-fold) in breast IDC tissues (0.86 ± 0.39) versus fibroadenoma (4.88 ± 1.79), (*P* < 0.0001).

No significant correlation was observed between relative expression of decorin mRNA and tumor characteristics including ER, PR, HER2, KI67, PNI, microcalcification, tumor necrosis, tumor size, and lymph node status in the studied IDC tissues, As presented in Table 1. Similarly no significant correlation was observed between the circulating levels of decorin and tumor characteristics including ER, PR, HER2, PNI, tumor size, and lymph node status in the studied breast cancer patients but a significant correlation was observed between plasma decorin and ki67, tumor necrosis and microcalcification. The mean of plasma decorin in patients with positive ki67 and tumor necrosis was lower than its level in patients with negative ki67 and tumor necrosis tissues. Patients with breast microcalcification had significantly higher plasma levels of decorin than those without microcalsification.

Significant Decrease in Decorin Protein Abundance in Breast IDC Tissues Than Fibroadenoma Is Consistent With Decorin mRNA Levels

In order to find out whether reduction in decorin mRNA levels in breast cancer is accompanied by a similar reduction in protein abundance, we performed immunohistochemical (IHC) analysis on all fibroadenomas biopsies (n = 11) and an almost equal number of breast cancer tissues biopsies (n = 12). Some of low, medium and high decorin mRNA-expressing breast cancer biopsies were selected for IHC.

If stained, decorin was mainly distributed in tissues' ECM and was observed in stroma, while no staining could be observed in cancer cells or epithelial cells. As shown in Figure 3A. Dim staining could only be observed in some of the IDC sections despite the moderate and strong staining intensity of decorin observed in both, breast IDC and fibroadenoma tissue sections.

In order to compare the mean of decorin abundance between the two study groups, the intensity of decorin signals were digitized into a grayscale from which the mean gray value of the internal control and background was subtracted. Then, the values were categorized into four grades as following; no staining (value 0–10) weak staining (value 11–25), intermediate staining (value 26–40), and strong staining (value over 40). Based on that, the observed mean of decorin distribution signal in breast cancer patients was calculated as 33.04 ± 10.21 while 62.37 ± 14.85 is the scoring values assigned to the fibroadenoma by our pathologist. Statistical analysis confirmed significantly lower decorin abundance in breast IDC versus fibroadenoma (*P* < 0.0001) as shown in Figure 3B.

Similar to the decorin mRNA levels, there was no significant relationship between decorin protein levels in stained tissue biopsies and ER, PR, HER2, KI67, PNI, microcalcification, tumor necrosis, tumor size, and lymph

(A)

(B)
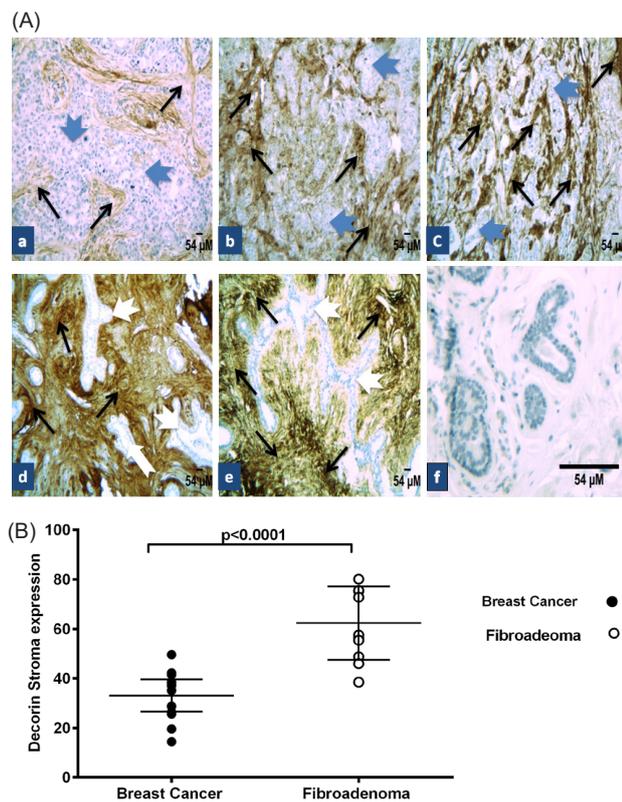
Figure 3. **Stromal Decorin Expression Assessed with Immunohistochemical Staining. (A)** Tissue serial sections visualized with immunohistochemical staining for decorin are presented. Weak (a), Intermediate (b) and strong (c) staining were observed in IDC tissue sections. Intermediate (d) and strong (e) staining for decorin in fibroadenoma tissue sections are also presented. As negative control (f) staining breast tissue sections without primary antibody was performed. Black arrows points at decorin staining in stroma. Epithelial glands in fibroadenoma are pointed with white arrows. Blue arrows mark invasive ductal carcinoma cells. Scale Bar = 54 μM is calculated with image J software. **(B)** The intensity of decorin signals were digitized into a grayscale from which the mean gray value of the internal control and background was subtracted. Mean gray value was estimated using ImageJ software. The values were categorized into four grades as follows: no staining (value 0–10), weak staining (value 11–25), intermediate staining (value 26-40) and strong staining (value over 40). The mean of stromal decorin expression in 12 breast IDC biopsy sections was estimated as 33.04 ± 10.21 (mean ± SD) while similar assessment assigned a 62.37±14.85 (mean ± SD) staining intensity to fibroadenomas. Applying *t* test confirmed statistical significant difference in staining intensity between the study groups with *P* value <0.0001.

node status of breast cancer patients.

Discussion

Based on the results of this study, plasma decorin levels did not seem to be applicable in distinguishing fibroadenoma from IDC tumors of breast despite the significant reduction in decorin expression within the microenvironment of breast carcinoma compared with fibroadenoma. To the best of our knowledge, this is the first study evaluating the diagnostic value of circulatory levels of decorin to distinguish benign from malignant tumors of breast.

Decorin has been proposed as a potential diagnostic biomarker in esophagus squamous cell carcinoma (ESCC)[26] and, recently, in urothelial carcinoma of bladder.[14] In an earlier study, I-Chen Wu and colleagues have shown, in a quite large number of patients (n = 275) that mean ± SD for plasma levels of decorin, calculated as 5.6 ± 3.6 ng/mL, is significantly lower than its level in similar number of healthy control individuals (7.8 ± 3.1 ng/mL; n = 295). With the use of the same ELISA kit, we detected similar levels of decorin in our patients' plasma. As opposed to the study by I-Chen Wu et al,[26] the correlation between plasma decorin and age was positive and significant in our breast cancer and fibroadenoma patients, both.

Recently, Appunni and colleagues reported the diagnostic value of plasma decorin in patients with bladder carcinoma when compared with healthy individuals.[14] Of interest in their data is the average of 20 fold lower circulating decorin levels they have detected compared to our study and Wu et al's study.[26] Assessing serum instead of plasma in that study might be one of the reasons for such low decorin levels. It has been reported that decorin has a binding tendency to circulatory fibrinogen.[32] Therefore, its major portion might get lost along with fibrinogen during serum preparation.

In order to evaluate whether or not the observed similar levels of plasma decorin in cancer and fibroadenoma patients in our study is due to lack of difference in the tissue levels of decorin, tissue biopsies from each patient were studied. Our data on tumoral expression of decorin strongly supports the significant decrease in decorin at mRNA and protein levels for breast malignancy in agreement with several other published studies.[33,34] Such a comparison with breast tissue of healthy individuals is only ethically possible when breast biopsies from reduction mammoplasty cosmetic surgery are available. Due to several limitations, we could not access a reliable number of such biopsies during the course of this study.

Studies in the past three decades have strongly demonstrated the role of decorin in maintaining tissue integrity and its importance in tumor progression and metastasis.[16,35,36] To date, the prognostic value of tissue decorin expression in several types of breast carcinoma is very well documented.[22] Applicability of decorin-based therapeutic approaches in reducing tumor progression and metastasis has also been proven through several studies not only in breast cancer [37] but also in several other types of tissue malignancies.[38,39] However, there have only been a few studies which evaluated the potential role of tissue decorin in distinguishing breast malignancies from benign tumors in patients.[40,41] Yet, those studies resulted in conflicting outcomes.

Comparing malignant legions with their assumedly unaffected margins which has been performed in several valuable studies[33,42] neither necessarily represent the microenvironmental properties of breast in a healthy individual nor excludes the potential effect of background genetic and epigenetic variables that could be taken into account when a population-based study is performed. Therefore, we specifically looked at the tissue expression of decorin in IDC versus a common type of benign breast tumor (fibroadenoma) in two different groups of patients.

The common diagnostic route to breast cancer starting with clinical examination, ultrasound and mammography,

still to date, requires biopsy excision and analysis as the final decision making step towards an appropriate therapeutic approach. However several diagnostic markers which have been proposed with a potential to differentiate benign and malignant tumors or to determine tumor development stage, none has emerged into clinic yet. Serum levels of human cartilage glycoprotein-39 or chitinase-3-like protein 1 also known as YKL-40[43] as well as miR-21, miR-221 seem to be significantly elevated in breast cancer patients in comparison with carriers of benign tumors or healthy controls.[43,44] However, a recent meta-analysis has assigned a more prognostic than diagnostic value for YKL-40 as its overexpression, despite ethnicity, seems to contribute to increased tumor size and advanced stage in breast cancer.[45] Similarly, elevated miR-221 has been shown to be associated with enlarged tumor size and tumor cell migration.[46] Therefore, it can be used as a reliable biomarker for disease prognosis in certain types of breast cancers such as triple negative and basal like breast cancers and tumor grade determination.[44,47] Acting as an oncogene, miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor diagnosis.[48] MiR-21 has also been shown to be a suitable candidate in differentiating breast cancer and fibroadenoma with less power compared to miR-221.[44] Recent meta-analysis strongly valued the predictive role of miR-21 for patient survival and possibly for early diagnosis.[49] However, the later requires further studies for a robust conclusion.

With specific regard to fibroadenoma, sequence analysis of the MED12 gene within the tumor lump has been shown to be a potential distinguishing factor between fibroadenoma and other types of breast tumors and a predicting factor for recurrent fibroadenoma. MED12 mutations, if occur in epithelial cells, are proposed to drive breast cancer whereas similar mutations in stroma lead to fibroadenoma as a result of variable communications with female hormones.[50] Based on our data, here we propose a similar role for the mRNA or protein levels of decorin to be analyzed on tissue biopsies. In conclusion, we have shown that tissue decorin is a reliable marker, unaffected by patient age and disease stage, to differentiate IDC from fibroadenoma. However, plasma decorin does not seem to have diagnostic value in this regard.

Authors' Contribution
ZF and TSH wrote the manuscript. ZF, RR, TSH and AB contributed to the study design and data analysis. ZF, RR, and TSH performed/analyzed experiments. SM performed statistical analysis and helped with drafting the manuscript. FS, FJah, PB and ShA provided surgical tissue specimens. AJ and FJad carried out histological examination. REZ contributed to patients' diagnosis. All authors read and approved the final manuscript.

Conflict of Interest Disclosures
The authors have no conflicts of interest.

Ethical Statement
The study was conducted in accordance with the Helsinki Declaration and approved by Zanjan University of Medical Sciences Ethics Committee (ZUMS.REC.1392.121. Nov. 2013).

Informed consent
Informed consent was obtained from all individual participants included in the study.

Availability of Data and Materials
The data that support the findings of this study are available from the corresponding authors on reasonable request.

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