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A STUDY OF STROMAL CD34 AND SMOOTH MUSCLE ACTIN IMMUNOEXPRESSION IN BENIGN AND MALIGNANT BREAST LESIONS AT A TERTIARY CARE CENTER IN NORTHERN INDIA: A CROSS-SECTIONAL STUDY.

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Abstract

Background

This study aimed to explore the distribution of CD34 and smooth muscle actin (SMA) positive myofibroblasts within the stroma of different benign and malignant breast lesions.

Material and methods

A total of 98 individuals with breast specimens acquired by surgery or biopsy were studied histopathologically and immunostained for CD34 and SMA using the avidin-biotin-peroxidase method. Appropriate comparisons among the distribution of CD34 and SMA along with statistical analysis were done to compare the sensitivity and specificity of each marker regarding the identification of benign and breast diseases.

Result

The histopathological findings included non-proliferative breast diseases (fibroadenoma, fibrocystic disease, phyllodes, gynecomastia, fibroadenosis), proliferative changes with and without atypia, carcinoma in situ, and invasive breast carcinoma. CD34 was positive in 98.27% of benign and negative in 92.37% of malignant breast diseases. SMA was strongly positive in 6.89% of benign and 80% of malignant breast diseases. In both DCIS and invasive breast carcinomas, there was a consistent loss of CD34 expression alongside a rise in SMA positivity. The sensitivity and specificity of CD34 stromal expression were 98.27% and 92.5%, respectively, while for SMA, they were 87.5% and 86.2%, respectively.

Conclusion

The combined assessment of CD34 and SMA holds promise as a diagnostic tool for recognizing the differences between benign and cancerous breast lesions. However, relying on either marker alone does not yield sufficiently distinct results.

Recommendations

Future studies should include detailed correlation analysis between CD34 and SMA expressions in breast fibroblasts to provide a more comprehensive understanding of their diagnostic value in distinguishing benign from malignant breast lesions.

Keywords: *CD34, Smooth muscle actin, Breast lesions, Diagnostic markers, Histopathological analysis.*
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Introduction

Breast cancer stands as the most common carcinoma and the second most prevalent cancer type among women worldwide [1]. Extensive research has delved into various facets of breast carcinoma, spanning epidemiology, diagnosis, novel treatment modalities, and prognostic factors. Consequently, there is a growing imperative to deepen the understanding of breast cancer's molecular and cellular underpinnings, leveraging this knowledge for targeted, molecular-based therapeutic interventions. CD34 and SMA are among the numerous markers proposed for diagnosing breast lesions, with studies suggesting an inverse correlation between CD34 expression and myofibroblastic differentiation [2-4].

The stromal microenvironment in neoplasms diverges significantly from that of normal cells, notably marked by an abundance of activated fibroblasts termed myofibroblasts (MFS) or cancer-associated fibroblasts (CAFs) within the tumor microenvironment (TME) [5,6]. Latest investigations have linked prognosis to the presence of MFS in the neoplastic stroma, highlighting the potential significance of stromal CD34 loss (CD34−) and

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α-SMA gain (α-SMA+) as indicators of tumor invasiveness in breast carcinoma [7-10]

CD34, a transmembrane glycoprotein expressed by various cells including hematopoietic stem cells, mesenchymal cells, and endothelial cells, is believed to modulate cell adhesion and signal transduction. According to some studies, CD34 fibrocytes are thought to be drawn from the bloodstream and enter tissue injury sites in breast lesions [11]. The loss of CD34 fibrocytes features stromal alteration associated with invasive carcinomas of the breast.[12, 13]

On the other hand, alpha-smooth muscle actin (α-SMA) is predominant in vascular SMC (smooth muscle cells) and contributes significantly to fibrogenesis. Activated myofibroblasts undergo a transition from proliferation to synthesizing abundant extracellular proteins [14].

The CD34-positive fibroblast exhibits multidirectional mesenchymal differentiation in many organs [15], with suggestions of an inverse association between CD34 expression and myofibroblastic differentiation [16,17]. The hypothesis regarding stromal CD34 loss and SMA myofibroblastic features as prerequisites for tumor invasiveness remains contentious, with some studies noting CD34 fibrocyte disappearance in invasive carcinoma stroma devoid of SMA myofibroblastic-like cells [11]. To address these discrepancies, the study aims to evaluate the distribution and existence of smooth muscle actin- and CD34-positive cells in the stroma of breast tumors that are benign and malignant.

Materials and methods

Study design

A prospective cross-sectional study.

Study setting

The proposed study was carried out on patients attending surgery outpatient at a tertiary care center spanning from May 2023 to June 2024.

Participants

The present study was conducted on 98 cases of breast carcinomas.

Inclusion criteria

Inclusion criteria had clinically suspected cases of benign and malignant lesions of the breast of all ages and patients providing consent.

Exclusion criteria

Inadequate and autolyzed samples were eliminated from the study. Then every slide was stained for mouse monoclonal antibody CD34 and SMA and examined.

Sample size

To calculate the sample size for this study, the following formula was used for estimating a proportion of a population:

$$n = \frac{Z2 \times p \times (1-p)}{E2}$$

Where:

- n = sample size
- Z = Z-score corresponding to the desired level of confidence
- p = estimated proportion in the population
- E = margin of error

Bias

To avoid result bias, the same slides were observed by two different observers.

Data collection

After taking informed and written consent, histopathological examination and immunohistochemistry of the breast specimens received in the department were analyzed. The data were recorded on a pre-formed questionnaire.

Procedure

Positive control was used for every antibody, to eliminate the possibility of wrong interpretation. The slides were stained by the same technician and similar reagents were used. For the comparative assessment of CD34 and SMA immunoreactivity, the stroma adjacent to the cancerous breast tissue and showing intraductal carcinoma were taken into account as separate entities.

Antibody against	Source	Clone	Code Number	Antigen retrieval	Incubation period with primary Antibody	Positive Control
CD34	BioSB	QBEnd 10	BSB5225	Microwave pH=6.0,20min	30-60 minutes	Blood vessel endothelium
SMA	BioSB	BSB15	BSB5029	Microwave pH=6.0,20min	30-60 minutes	Normal gland myoepithelial layer

The study compared CD34 fibrocytes and SMA reactive myofibroblasts in the stromal areas around the tumor and the surrounding tumor-free mammary tissue of the carcinoma. To support the semi-quantitative assessment of CD34 and SMA in stromal tissue, CD31 and p63 were also measured. Under optic microscopy, the slides were inspected with an Olympus CX21i microscope. Three distinct cell types were seen, together with their location,

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density, and immunostaining pattern, as the immune-reactivity of CD34 and SMA was semi-quantitatively assessed. Focal positivity was observed in some cases of the three categories of fibroblast reactivity for CD34 and αSMA: positive, negative, or decreased. Two impartial observers assessed the percentages. The study assessed the correlation between staining patterns and pathology for each duct-lobular unit because numerous sections contained ducts and lobules displaying a range of histopathological characteristics.

Statistical analysis

Sensitivity and Specificity were calculated. A statistical method such as the calculation of p-value and Z test was employed to find out the significance of the study. A statistician's help was sought for the interpretation of results.

Ethical considerations

The institutional ethical committee granted ethical approval.

Results

Table 1. Distribution of cases according to histo-pathological diagnosis of breast lesions

S. No.	Histopathological diagnosis	Number of cases	Percentage (%)	Total
A.	**Non-proliferative breast changes**			53.05
	Fibroadenoma	36	36.73	
	Fibrocystic disease	08	8.16	
	Benign Phyllodes	02	2.04	
	Gynaecomastia	02	2.04	
	Fibroadenosis	01	1.02	
	Chronic Non-Specific Mastitis	01	1.02	
	Fibroadenoma with Keratinous Cyst	01	1.02	
	Duct Papilloma	01	1.02	
B.	**Proliferative disease without atypia**			
	Usual Ductal hyperplasia	03	3.06	3.06
C.	**Proliferative disease with atypia**			
	Atypical Ductal Hyperplasia	03	3.06	3.06
D.	**Carcinoma in situ**			
	Ductal carcinoma in situ (DCIS)			
	− High-grade DCIS	02		
	− Intermediate grade DCIS	01		
	− Low-grade DCIS	01		
	Total	04	4.08	4.08
E.	**Invasive carcinoma**			
	-Infiltrating ductal carcinoma	27	27.55	
	− Infiltrating lobular	02	2.04	
	− Medullary carcinoma	03	3.06	
	− Mucinous carcinoma	02	2.04	
	− Leiomyosarcoma	01	1.02	
	− Hemangioendothelioma	01	1.02	
	Total number of cases	98	100%	36.75

The histopathological details of the 98 subjects are given in Table 1. Among malignant breast cases, Infiltrating Ductal Carcinoma was the most common (27 cases; 27.55%) histological type. The average age was 23.5 years for benign lesions. Out of 98 cases, 95 cases (96.93%) were females, and 3 cases (3.06%) were male patients.

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Table 2: SMA and CD34 expression in stromal cells of benign breast lesions

Histology	SMA Positive	SMA+/-	SMA Negative	CD34 Positive	CD 34+/-	CD34 Negative
Fibroadenoma (36)	0	4	32	35	1	0
Fibrocystic DS (8)	2	0	6	8	0	0
Phyllodes (2)	1	0	1	1	1	0
Gynaecomastia (2)	0	0	2	2	0	0
Keratinous cyst (1)	0	0	1	0	1	0
Duct papilloma (1)	0	0	1	1	0	0
Fibroadenosis (1)	0	0	1	1	0	0
Chronic Nonspecific Mastitis (1)	0	0	1	1	0	0
Usual ductal hyperplasia (03)	0	0	3	3	0	0
ADH (03)	1	0	2	2	0	1
Total	4	4	50	54	3	1

In Table 2, the analysis of CD34 expression in benign breast lesions revealed that out of 58 benign cases studied, stromal immunoreactivity was present in 57 cases (98.27%), while only 1 case (1.72%) showed negative CD34 expression. The majority of histologically assessed cases were fibroadenomas, with 35 out of 36 cases (97.22%) displaying strong CD34 stromal positivity. These CD34-positive stromal cells within fibroadenomas exhibited a dendritic or stellate morphology and were more densely populated compared to normal breast stroma. Notably, a central concentration of fibroblasts was observed, along with a progressive loss of positive reaction around collapsed ducts.

Within the nearby normal breast tissue, glandular ducts and acini in the intralobular and interlobular regions were surrounded by CD34-positive stromal cells. In contrast to the interlobular stroma, which had a lesser density of positive CD34 stromal cells and positively stained vascular endothelium, the intralobular stroma exhibited a larger intensity of positive reactivity. Any CD34 reactivity in epithelial cells was not observed. Of the two Phyllodes instances, one had reduced staining of spindle cells next to epithelial components while the other had diffuse CD34 staining in the stroma. A concentric, circular pattern of CD34-positive stromal cells was seen in patients with Usual Ductal Hyperplasia. When CD34 expression in malignant breast lesions was analyzed, it was shown that the majority of malignant cases (37 out of 40, 92.5%) had lost CD34 expression in the stroma, with its expression only present in the tumoral vascular endothelium. Positive cells for CD34 were found in a stroma zone that was segregated from the surrounding adipose tissue and the tumor.

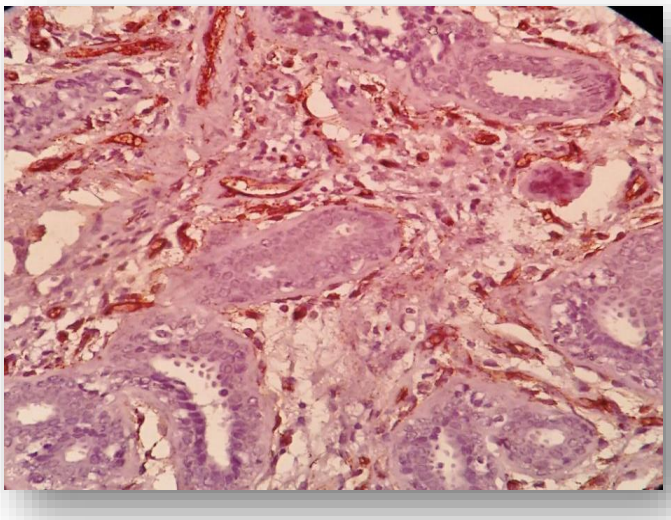

Figure 1: 55Immunohistochemical expression of CD34 in Fibrocystic disease showing strong stellate /dendritic positivity in stroma of fibrocystic disease breast

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Fibroblasts surrounding ducts with DCIS showed a varied pattern of expression in the examination of CD34 expression in DCIS. The periductal stroma of patients with high-grade DCIS revealed reduced CD34 expression, whereas that of patients with intermediate-grade DCIS showed a loss of CD34 expression. Around low-grade DCIS foci, CD34 expression was found to be retained.

Regarding the expression of SMA in various breast lesions, most benign lesions such as fibroadenoma, usual ductal hyperplasia, and papilloma exhibited positive SMA only in the myoepithelial cell layer and media of vessels. However, a minority of cases showed focal rare positive cells for SMA in the stroma, including 4 cases of fibroadenoma (11.11%), 2 cases of fibrocystic disease (25%), and 1 case of atypical ductal hyperplasia (33.33%).

Table 3: SMA and CD34 expression in malignant breast lesions

Histology	SMA Positive	SMA+/-	SMA Negative	CD34 Positive	CD 34+/-	CD34 Negative
Ductal carcinoma in situ (4)	2	1	1	**1**	**1**	**2**
Ductal carcinoma (27)	25	0	2	**0**	**0**	**27**
Lobular carcinoma (2)	1	0	1	**0**	**0**	**2**
Medullary carcinoma (3)	3	0	0	**0**	**0**	**3**
Mucinous carcinoma (2)	0	2	0	**0**	**0**	**2**
Leiomyosarcoma (1)	1	0	0	**0**	**0**	**1**
Hemangioendothelioma (1)	0	0	1	**1**	**0**	**0**
Total	32	3	5	**2**	**1**	**37**

Table 3 shows that 35 cases (87.5%) of the 40 cases of malignant breast lesion cases, including infiltrating ductal carcinoma, showed a loss of CD34 expression and an increase of SMA expression. SMA-positive myoepithelial cells formed a continuous layer around components of the Terminal Duct Lobular Unit (TDLU) in neighbouring normal breast tissue. SMA-reactive myofibroblasts were not seen in the stroma of normal breast tissue, but SMA was found in the walls of muscle arteries as well.

Analyzing SMA expression in malignant breast lesions, the majority of invasive carcinomas (35 out of 40 cases, 87.5%) demonstrated consistent loss of CD34 expression in fibroblasts and gain of SMA expression in the stroma. Faint staining for CD34 was discernible in small-caliber blood vessels within the stroma. In mucinous carcinoma, weak positivity for SMA was noted.

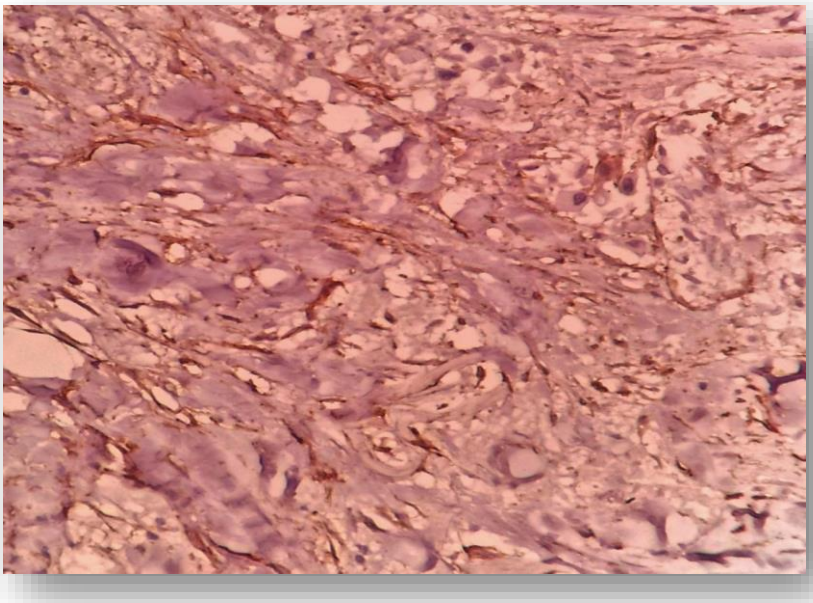

Figure 2: Immunohistochemical expression of SMA in Infiltrating ductal Carcinoma showing strong positive expression of myofibroblast stroma

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A variable expression pattern was observed in fibroblasts surrounding ducts with DCIS. Among 4 cases of DCIS, both high-grade ductal carcinoma in situ cases displayed loss of CD34 expression within the periductal stroma and gained SMA positivity. One intermediate DCIS case showed reduced CD34 expression within the periductal stroma alongside emerging SMA expression, while another low-grade DCIS case retained CD34 expression around foci of low-grade DCIS and lacked SMA expression.

Table 4: Evaluation of CD34 & SMA in benign vs. malignant lesion of breast

	CD34		SMA	
Lesion	**Positive**	**Negative**	**Positive**	**Negative**
Benign	57	1	8	50
Malignant	3	37	35	5
Chi^2	78.392		49.277	
P value	<0.0001		<0.0001	

The assessment of CD34 and SMA expression in benign and malignant breast tumors is shown in Table 4, with chi-squared test statistical analysis applied. The expression of SMA and CD34 varied significantly ($p<0.0001$) between benign and malignant breast tumours, according to the data.

Moreover, a statistically significant distinction was detected in the stromal expression of CD34 and SMA between benign and malignant breast tumours when comparing their correlation ($p<0.0001$). Interestingly, in high-grade DCIS and infiltrating breast cancer, there was a negative connection between SMA expression and CD34 expression.

When it comes to detecting benign and malignant breast lesions, CD34 stromal expression showed 98.27% sensitivity and 92.5% specificity. SMA, on the other hand, demonstrated 86.2% specificity and 87.5% sensitivity for the same objective.

DISCUSSION

The patients included in the study ranged from 15 to 70 years of age, with the largest proportion (48.98%) falling in the 21-40 years age group. Only 12 cases (12.24%) were below 20 years old. Similar findings were observed by Sharma R et al [18], who noted a majority of cases in the 21-40 years age group in their study of 100 cases of breast lumps.

The higher incidence of malignancy (40.82%) observed in the study compared to others can be attributed to this being a prominent referral center in the eastern part of Uttar Pradesh, adjacent to areas of Nepal and Bihar. Additionally, a significant portion of the patient population is illiterate or poorly educated, with limited awareness of breast cancer. These factors, combined with limited available data, could explain the elevated incidence of carcinoma observed in this region.

The significance of molecular markers such as CD34 and SMA immunoexpression as stromal markers in benign and malignant breast lesions has been explored in only a few studies. As a result, their importance and relevance need further extensive validation and study.

Consistent with the findings, stromal-positive CD34 fibroblasts are associated with benign lesions. In the study, 98.27% of cases had CD34-positive fibroblasts visible, and the majority of benign cases had Fibroadenoma histologically detected. Out of 36 Fibroadenoma cases, 35 (97.22%) exhibited strong CD34 stromal positivity, as depicted in Figure 1. These CD34-positive stromal cells within Fibroadenoma displayed a dendritic or stellate morphology, with a higher density compared to normal breast tissue stroma. Additionally, a central concentration of fibroblasts was noted, along with a progressive loss of positive immunoreactions around collapsed ducts. The study findings are similar to studies by Cimpean AM et al [12], Fadil SA et al [19], and Chauhan et al [13].

Similarly, Kuroda N et al [20] investigated 33 benign breast lesions and found CD34-positive stromal cells in 29 of these lesions (87.87%), further supporting the correlation of CD34 expression with benign lesions.

In one of the two cases of Benign Phyllodes tumors in the study, diffuse CD34 was staining in the stroma, whereas the other case showed reduced staining of spindle cells adjacent to the epithelial element. Interestingly, the CD34 density was higher than in Fibroadenoma cases. These data are in line with the results published by Fadil SA et al. [19], who also observed that in certain cases of benign Phyllodes tumours, spindle cells next to the epithelium expressed less CD34. These results imply that interactions between mesenchymal and epithelial cells may affect CD34 expression.

Further supporting the concept of epithelial-mesenchymal interaction in Phyllodes tumors, there are findings indicating that p53 expression in Phyllodes tumors is primarily observed in the stroma immediately adjacent to the epithelium, as reported by Millar EK et al [21]. These findings collectively indicate a complex interplay between epithelial and mesenchymal components in the context of Phyllodes tumors.

In the case of Usual Ductal Hyperplasia, the study observed a circular, concentric pattern of CD34-positive stromal cells, a finding consistent with the study by Cimpean AM et al [12]. Similarly, Hua X et al [22]

detected CD34 protein expression in the cytoplasm of stromal fibroblasts in a high percentage (94.44%) of cases of usual ductal hyperplasia. In the study, 100% CD34 expression was seen in all three cases of usual ductal hyperplasia.

Regarding Smooth Muscle Actin (SMA) expression in benign lesions, including usual ductal hyperplasia and papilloma, SMA was primarily positive only in the myoepithelial cell layers and media of vessels. However, in a few cases (4 out of 72) of fibroadenoma, focal or rare positive cells of smooth muscle actin were observed in the stroma along with CD34 positivity.

Both CD34 and SMA antibody-positive fibroblasts and myofibroblasts were seen in the hypercellular stroma of the two benign Phyllodes tumour patients. Interestingly, in these instances, the density of CD34-positive cells was greater than that of Smooth Muscle Actin-positive cells. These findings shed light on the diverse ways that SMA and CD34 are expressed in different types of benign breast tumours.

Similar to the study, previous research by Cimpean AM et al [12], Chauhan et al [13], Fadil et al [19], Hao Yi et al [23], and Kuroda et al [20] also reported the absence of SMA positive cells in the stroma of normal breast tissue, except for myoepithelial cells. This consistency in findings underscores the limited presence of SMA-positive stromal cells in benign and normal mammary tissue.

Around DCIS, fibroblasts showed the most varied expression pattern. Compared to intermediate or low-grade lesions, high-grade lesions were more likely to exhibit the loss of CD34 expression and the gain of SMA expression. The change in breast carcinogenesis from DCIS to infiltrating ductal carcinoma is a crucial but little-understood stage. The results are consistent with research by Catteau X et al [9] and Chauhan et al [13], which show that fibroblast CD34 expression is lost in invasive carcinoma cases, including micro-invasive cases, and in a considerable number of DCIS cases, especially in high-grade lesions that have a higher propensity to invade. Around DCIS foci, there was a gradual rise in Smooth Muscle Actin expression that was inversely linked with this reduction of CD34 expression.

A possible connection between CD34 loss and invasive potential is suggested by Chauhan et al.'s [13] observation that CD34 expression is lost in Atypical Ductal Hyperplasia but not in areas where Lobular Carcinoma is visible in situ. As previously shown in phyllodes tumours, the localized loss of CD34 around ducts carrying DCIS, while maintaining expression around nearby normal breast glands, highlights the significance of epithelial-mesenchymal interactions in regulating CD34 expression. It is important to comprehend the reasons that determine the loss of CD34 expression in DCIS because this loss is not present in all cases, indicating that the neoplastic epithelium has different functional states that affect its capacity for invasion.

Regardless of the histological type, all invasive breast lesions (100%) in the investigation showed total loss of stromal CD34 fibrocytes (Figure 2). SMA expression was consistently acquired alongside this loss of CD34 expression. As a result, a strong inverse relationship ($p<0.001$) between the expression of SMA and CD34 in breast infiltrative lesions was discovered. These findings point to a negative correlation between malignant breast tumours and CD34 fibrocytes. These results are consistent with earlier research conducted by Kuroda et al. [20], Chauhan et al. [13], and Cimpean AM et al. [12].

Similarly, Ramaswamy et al. [11] have shown that, in the majority of instances, the presence of alpha-SMA-positive myofibroblasts and the lack of CD34-positive stromal cells is suggestive of malignancy. It is noteworthy, nevertheless, that Barth et al. [11] and Chauhan et al. [13] reported the disappearance of CD34-positive stromal cells in the breast stroma of patients with invasive ductal carcinoma; however, these studies excluded patients with invasive lobular or medullary carcinoma.

In the present work, the statistical analysis showed substantial differences ($p<0.001$) in the expression of CD34 and SMA between infiltrating cancer and benign lesions. In particular, there was a substantial difference ($p<0.001$) between the expression rate of SMA in benign lesions and malignancies, while there was a substantial increase ($p<0.001$) in the expression grade of CD34 in benign breast tissue compared to malignancies.

There is data in the medical literature about the sensitivity and specificity of CD34 and SMA stromal expression. The results of the investigation showed that the sensitivity and specificity of SMA were somewhat lower at 87.5% and 86.2%, respectively, but the sensitivity and specificity of CD34 were 98.27% and 92.5%, respectively. The potential diagnostic use of CD34 and SMA in differentiating between benign and malignant breast tumours is highlighted by these results; however, additional research is required to validate and build upon these findings.

Generalizability

The findings from this study are potentially generalizable to other tertiary care centers with similar patient demographics, given the robust sample size and standardized immunohistochemical methods used. However, variations in patient populations and local healthcare practices should be considered when applying these results broadly. Further multicenter studies are recommended to validate and enhance the generalizability of these diagnostic markers in diverse clinical settings.

Conclusion

As observed in DCIS, the breast stroma's change from CD34-positive fibrocytes to SMA-positive myofibroblasts may be a sign that cancer cells are about to enter the stroma. This shift, wherein the stroma showed a total loss of CD34 fibrocytes and gained expression of SMA, was uniformly observed in all invasive breast tumours. Although there were a few outliers, the findings suggest that, particularly in difficult instances, the

combined immunohistochemistry expression of SMA and CD34 may be useful in diagnosing benign from malignant breast tumours. To precisely understand the function of CD34 and SMA-positive stromal cells in breast lesions and their clinical implications, more investigation is needed.

Recommendations

Future studies should include detailed correlation analysis between CD34 and SMA expressions in breast fibroblasts to provide a more comprehensive understanding of their diagnostic value in distinguishing benign from malignant breast lesions.

List of abbreviations

SMA: Smooth Muscle Actin
DCIS: Ductal Carcinoma In Situ
MFS: Myofibroblasts
CAFs: Cancer-Associated Fibroblasts
TME: Tumor Microenvironment
SMC: Smooth Muscle Cells
TDLU: Terminal Duct Lobular Unit
α-SMA: Alpha-Smooth Muscle Actin

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Conflict of interest

The authors have no competing interests to declare.

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