IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 21, Issue 2 Ser.1 (February. 2022), PP 37-43
www.iosrjournals.org

A study of CD34 antigen expression in benign and malignant breast lesions in patients attending RIMS, Ranchi.

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Abstract
Background :*Breast cancer is the most frequent cancer among women. In recent years many markers have come up as a reliable tool for determining the prognosis of the patients with breast carcinoma. Recently CD34 has also been suggested as a diagnostic marker in breast lesions. It has been suggested that CD34 may be related to invasive potential. This study is being undertaken to study the expression of CD34 in breast lesions and to relate its extent of expression in various breast pathologies.*
Materials and methods: *this retrospective study was conducted on histopathological specimens from female breast. Post surgical specimen including biopsies and mastectomies were fixed in 10% neutral buffered formalin. Initial diagnosis were made by H&E stained tissue sections. Sections from the same block were subjected to immunohistochemical staining using monoclonal antibody to CD34 antigen.*
Result: *Benign lesions had greater expression of CD34antigen while most of the malignant ones had lost or had minimal expression.*
Conclusion:*CD34 antigen has a potential for being used as a marker in the diagnosis of breast carcinoma.*
Keywords: *breast, benign, malignant, CD34*

Date of Submission: 20-01-2022 Date of Acceptance: 03-02-2022

I. Introduction

Breast carcinoma is the second most common carcinoma in the world and by far the most frequent cancer among women. It is also the second most common cancer in women in rural areas[1]. Ample research has been undertaken on all aspects of breast carcinoma including epidemiology, diagnosis , new methods of treatment and prognostic factors. Among many other markers in recent years CD34 has also been suggested as a diagnostic marker in breast lesions. It has been suggested that there is an inverse relation between CD34 expression and myofibroblastic differentiation[2]. CD34 is a transmembrane glycoprotein that is involved in the modulation of cell adhesion and signal transduction and is expressed by mesenchymal cells at various sites, including the normal mammary stroma.[3] Loss of CD34 by mesenchymal cells has been described in several situations where there is malignant transformation of the mesenchymal population . Malignant phyllodes tumor of the breast exhibit lower levels of CD34 than benign phyllodes tumor or fibroadenoma [4] and CD34 is lost in sarcomas arising within CD34 positive dermatofibrosarcoma protuberans [5]

II. Materials And Methods

Study was conducted in Department of Pathology, Rajendra Institute of Medical Sciences, Ranchi. Cases comprised of the histopathological specimens received in the Department of Pathology, operated in Rajendra Institute of Medical Sciences, Ranchi. The study was conducted over a period of one year from June 2019 to May 2020. A total of 52 cases were studied. There were certain inclusion and exclusion criteria for the cases to be selected for study, which were as follows:

INCLUSION CRITERIA:

1. Age group 20-70 years.
2. Only samples with definite histopathological diagnosis were considered.

EXCLUSION CRITERIA:

1. Age <20 and >70
2. Male breast

SPECIMEN SELECTION AND HANDELING:

Post surgical specimens included biopsies and mastectomies. They were fixed in 10% neutral buffered formalin. Gross examination of the specimen was performed and sections were taken from the representative areas. They were processed by an automatic tissue processor. On all these these cases which were initially diagnosed by Hematoxylin and Eosin (H&E) CD34 immunostaining was performed.

Procedure for CD34 immunostaining

Immunohistochemistry was done by indirect immunoperoxidase technique (Novocastra liquid mouse monoclonal reagents by Leica Microsystems). This was carried out by employing the following steps-

1. Cut tissue sections of 3 microns and collect them on positively coated slides.
2. Bake sections for 1 hour at 60.c prior to test
3. Immerse the warm slides in xylene for 5 min- 2 times
4. Allow the sections to soak in wash buffer for 2 minutes in a staining jar
5. Rehydration – 3 min each (Absolute alcohol, 70% alcohol, 50% alcohol)
6. Antigen retrieval (microwave technique) – AR in citrate buffer-

Cycle 1: 85.c for 5 min or 500 watts for 5 min Cycle 2: 98.c for 15 min or 750 watts for 15 mins

7. Allow the slides to cool at room temperature for 15 min.
8. Wash with wash buffer for 3 times with a gap of 30 secs for each wash
9. Incubate the slides in Peroxidase block – 10 min at room temperature
10. Wash with wash buffer for 3 times with a gap of 30 secs for each wash
11. Incubate the slide in protein block – 10 min at room temperature
12. Wipe the slides properly and incubate with primary antibody at room temperature 1 hr.
13. Wash the slides in wash buffer for 3 times with a time gap of 30 secs for each wash
14. Incubate with super enhancer – 20 min at room temperature
15. Wash the slides 4 times with wash buffer with a time gap of 30 sec for each wash
16. Incubate with HRP polymer – 30 min at room temperature
17. Wash the slides 4 times with wash buffer with a time gap of 30 secs for each wash
18. Apply freshly prepared DAB (Diamino benzidine) solution and incubate for 7- 10 min.
19. Wash with wash buffer 4 times with a time gap of 30secs for each wash
20. Counterstain the slide with hematoxylin – 3 min
21. Wash the slide DI water for 3 times with a time gap of 30 secs for each wash
22. Dehydration – 2 min each (50% alcohol, 70% alcohol, absolute alcohol)
23. Xylene (1) – 5min
24. Xylene (2) – 5min
25. Mount in DPX properly

METHOD OF EVALUATION OF CD34 IMMUNOSTAINING

The representative sections from all the cases were studied. Each section was examined and the number of duct/ lobular units was identified. The grading was done using the method suggested by Catteau et al (2013). The sections were evaluated at high power (400 X microscopic field; objective 40 X, eyepiece 10X), assuming that a high power microscopic field harboured 100 stromal cells.

Grading was done from 0 to 3+, where

0 : upto 5% stromal cells immunoreactive
1+ : >5 and upto 25% stromal cells immunoreactive
2+ : >25 and upto 50% stromal cells immunreactive
3+ : > 50% stromal cells immunoreactive

Assuming that a high power microscopic field harboured 100 stromal cells.(Catteau et al, 2013).
The staining of endothelial cells in blood vessels was taken as internal control (Cimpean et al,2005) Grade 0 was interpreted as complete loss of CD34
Grade 1 was interpreted as reduced expression,
While grade 2 and 3 were interpreted as retained expression of CD34

III. Result

This study was carried out on 52 histopathological specimens of benign and malignant breast lesions received in Department of Pathology, Rajendra Institute of Medical Sciences, Ranchi, Jharkhand
Distribution of cases:
A total of 52 breast lesions were divided into the following broad categories:

1. Non neoplastic- 13 cases (24.98%)

2. Neoplastic: 39 cases (74.97%)- Benign- 15 cases
Malignant- 24 cases

TABLE 1. HISTOPATHOLOGICAL DISTRIBUTOIN OF CASES

NATURE	**LESION**	**NO. OF CASES**
NON NEOPLASTIC 13cases (24.98%)	Fibrocystic disease	7
	Chronic mastitis	2
	fibroadenosis	2
	Ductal adenosis	1
	Sclerosingadenosis	1
NEOPLASTIC 39 cases (74.97%)	**BENIGN: 15 cases (30.76%)**	
	Fibroadenoma	10
	Papilloma	1
	Benign phyllodes	4
	MALIGNANT: 24 cases (46.15%)	
	IDC	17
	ILC	2
	Metaplastic carcioma	3
	Malignant phyllodes	2
TOTAL		52

Each case was studied and CD34 staining was assessed in the interlobular and intralobular stroma. The changes in CD34 were found to be highly localized with separate areas in the same section showing completely different staining patterns.

TABLE 2. SHOWING THE EXTENT OF EXPRESSION OF CD34 IN THE STROMAL CELLS IN VARIOUS LESIONS

Histology	**% of TDLU expressing CD34**			
	0+	1+	2+	3+
Non-neoplastic lesions				
Fibrocystic disease	0%	0%	20%	80%
Chronic mastitis	0%	25%	70%	05%
Fibroadenosis	0%	0%	30%	70%
Sclerosing adenosis	0%	06%	64%	30%
Ductal adenosis	0%	08%	15%	77%
Neoplastic lesions- benign				
Fibroadenoma	0%	05%	12%	83%
Benign phyllodes	0%	10%	18%	72%
Intraductal papilloma	0%	50%	50%	0%
Neoplastic lesions- malignant				
Infiltrating ductal carcinoma	98%	2%	0%	0%
Invasive lobular carcinoma	97%	3%	0%	0%
Malignant phyllodes	83%	15%	02%	0%
Metaplastic carcinoma	95%	5%	0%	0%

In normal mammary tissue, CD34 positive stromal cells were observed around glandular ducts and acini in the intralobular area. However, the number of CD34 positive stromal cells was, low in the interlobular stroma. Majority of normal TDLU showed grade 3+ staining. CD34 expression was studied in the normal areas of breasts with lesions.

DISTRIBUTION OF CD34 IN VARIOUS BREAST LESIONS:

Non neoplastic lesions (13cases)

Cases with a diagnosis of chronic mastitis, fibroadenosis, ductal adenosis, sclerosing adenosis and fibrocystic disease were included in this category.

Cases with diagnosis of chronic mastitis showed many CD34 positive stromal cells present in the stroma surrounding the glandular ducts. Most of the TDLU showed staining of grade 2+.

The cases with diagnosis of fibrocystic disease, fibroadenosis and ductal adenosis were shown to have moderate to strong and diffuse pattern of staining . Grade 3 staining was found in 80% of TDLU of fibrocystic disease, 70% of TDLU of fibroadenosis and 77% TDLU of ductal adenosis.

Fibroadenoma (10 cases)

Fibroadenomas showed moderate to dense and diffuse stromal expression of CD34 . The staining was more marked in the interlobular stroma. 83% of TDLU showed grade 3+ staining 12% of the TDLU showed moderate immunostain.

Phyllode's tumor (6 cases)

The hypercelluar stroma of phyllodes tumor contained fibroblasts that were strongly positive for CD34 . Malignant phyllode's tumor had faint patchy expression of CD34 . The loss of CD34 was seen in 83% of TDLU in malignant phyllodes as compared to 0% cases of benign phyllode's.

Intradutal papilloma(1 case)

Ductal papilloma showed 1+ to 2+ grade staining in the stroma. They also showed accentuation of fibrovascular cores.

Malignant tumors (24 cases)

All the malignant tumors showed similar staining pattern with almost complete loss of CD34 expression in all the cases. Loss of CD34 expression was seen in 98% TDLU of infiltrating ductal carcinoma. Metaplastic carcinoma and infiltrating lobular carcinoma showed complete loss of CD34 expression in 95% and 97% of TDLUs respectively. Malignant phyllodes showed loss of CD34 in 83% of the TDLUs. The vascular endothelium stained strongly and was taken as internal control.

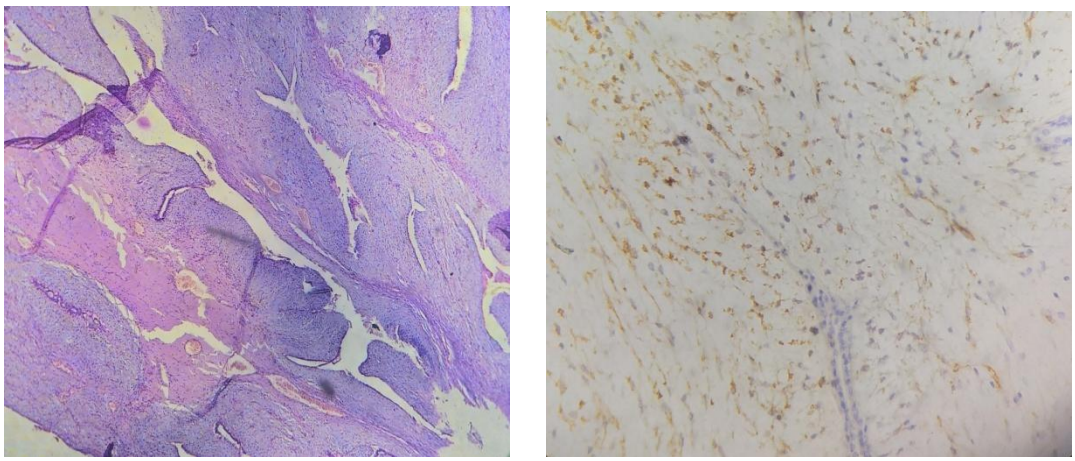

Fig 1.H&E staining 20x and CD34 immunostain 40x respectively- Benign phyllodes

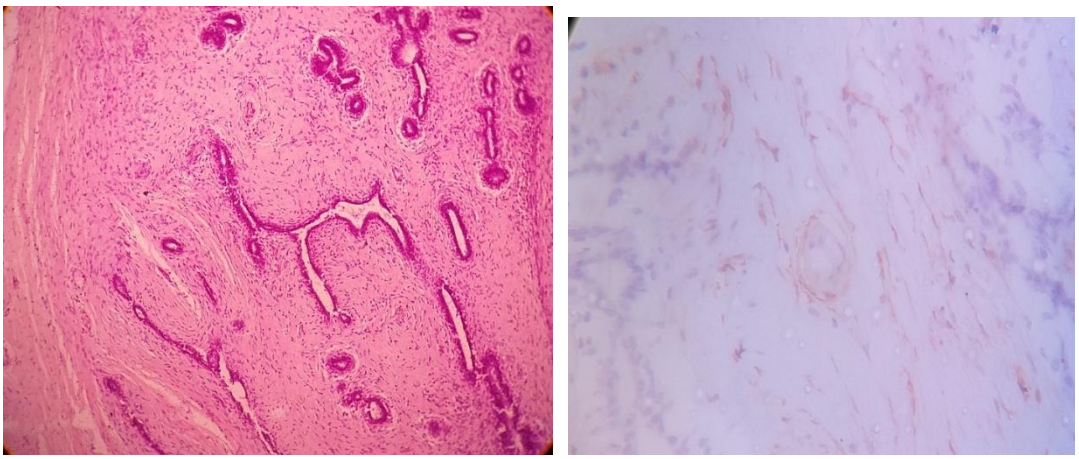

Fig 2. H&E staining 20x and CD34 immunostai n 40x respectively- Fibroadenoma

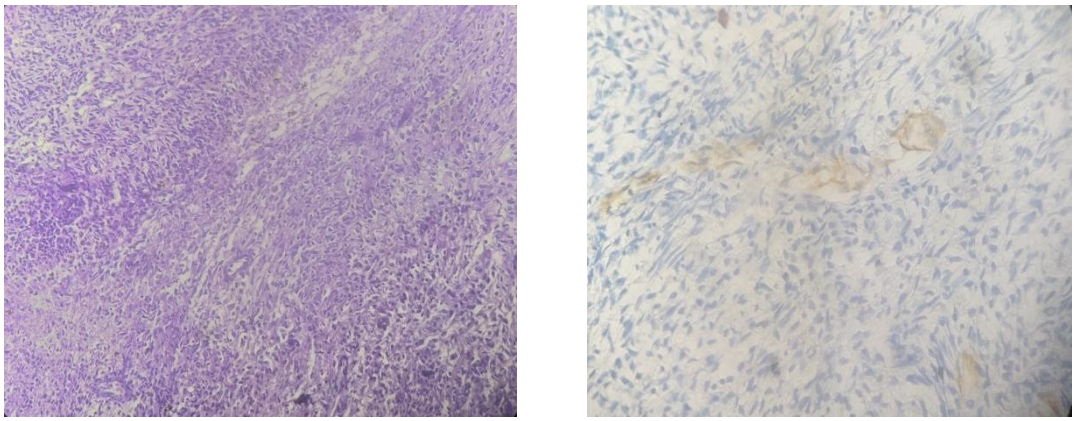

Fig 3. H&E staining 20x and CD34 immunostain 40x respectively- malignant phyllodes

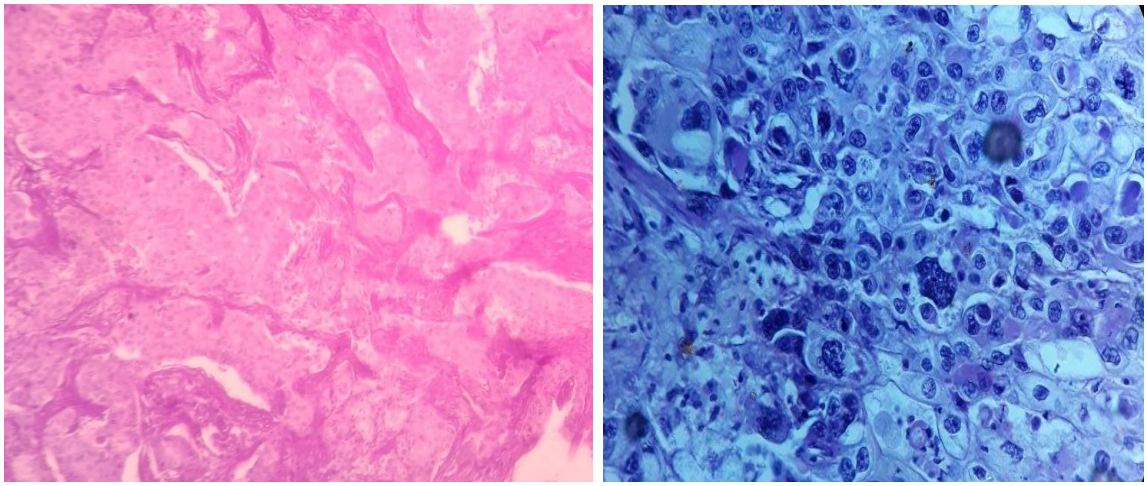

Fig 4. H&E stain 20x and CD34 immunostain 40x respectively- Metaplastic carcinoma breast.

IV. Discussion

Breast cancer continues to form a significant burden of disease worldwide as well as in India. Despite the current advances and ample studies undertaken in the field of breast cancer, it continues to exact a heavy toll on human life. Studies on newer methods for adequate and timely diagnosis, as well as treatment of breast cancer are the need of the hour.

CD34 has come up as a potential new marker for diagnosis of invasive breast cancer, as shown in various studies published earlier and it has also been demonstrated that the loss of CD34+ fibrocytes in breast stroma is specific for invasive breast cancer [2,6]. This new marker could add to the panel of various immunomarkers already in use in cases of breast cancer, eg., ER, PR, HER2/neu etc.

Many studies have been published till now regarding the expression of CD34 in various lesions [2,7]. However, there have been varying data and slight different results have been obtained by different authors. Nonetheless, there is a general consensus that CD34 appears to be a new and upcoming marker which can be useful not only in the diagnosis of breast cancer, but also can be a potential therapeutic target for breast cancer cases in the future. Keeping in mind the above facts, this study was done to evaluate the expression of CD34 in benign and malignant breast lesions and to determine whether the loss of CD34 is specific for invasive disease.

In our study the cases were divided into non neoplastic and neoplastic. Neoplastic cases formed the larger group with 39 cases (74.97%). Out of all the neoplasms, 46.15% (24 cases) were malignant. Overall, the largest category was of infiltrating ductal carcinoma (17 cases, 32.69%), followed by fibroadenoma (10 cases, 19.23%).

The non neoplastic cases (13 cases, 23.07% of total) included fibrocystic diaese (7 cases, 53.84%), two cases each of chronic mastitis and fibroadenosis (15.38% each), and one case each of ductala denosis and sclerosing adenosis (7.69%). Benign neoplasms (15 cases,30.76% of total) included fibroadenoma (10 cases, 66.66%), benign phyllodes tumor (4 cases, 26.66%) and one case of intraductal papilloma (6.66%).

Malignant neoplasms (24 cases, 46.15% of total) included infiltrating ductal carcinoma (17 cases, 70.83%), metaplastic carcinoma (3 cases, 12.5%) and two cases each of malignant phyllodes and infiltrating lobular carcinoma (8.33% each).

Table 3.Number and distribution of cases in previous studies. Figures in parenthesis indicate number of cases.

CATTEAU, 2013	**KURODA, 2005**	**CIMPEAN, 2005**	**CHAUHAN, 2003**	**BARTH, 2002**
Total- 48 cases: DCIS(20), DCIS Associated with IDC (12), IDC (16)	Total-64 cases Fibrocystic ds (12), Papilloma (4), Fibroadenoma (17), ILC(6), IDC(20), Invasive micropapillary Ca(5)	Total-112 cases Normal (5) Sclerosingadenosis(7), fibroadenoma(16), fibrocystic ds (11), phyllodes tm (1), DCIS(1), IDC(46), ILC(10), Squamous Cell type(5), Medullary ca(5), Mucinous ca(5), Papillary ca(5)	Total -135 Normal(10), Fibroadenoma (10) IDC(40), DCIS (55), Radial scar(20)	Total -58 Sclerosingadenosis(12), Fibroadenoma (7), phyllodes Tm(1), Microglandular Adenosis(1), Ductal hyperplasia(9), Tubular adenoma(1), IDC (31)

Most of the patients included in non-neoplastic category were of fibrocystic disease (53.84%). These patients ranged in age from 20 to 45 years with most of them in the age group of 40 to 45 years. Some researchers found the peak age of incidence for fibrocystic disease to be 40 to 44 years[8] .This study was conducted in the western population. However, Tiwari and Tiwari conducted a study on Indian females and reported that most of the patients of fibrocystic disease in their study (44.28%) were from third decade.[9]

Among the benign neoplasms, fibroadenoma formed the largest group (66.66% of cases). The age range for fibroadenoma was from 20 to 40 with most of the patients in the age group of 20 to 30 years. Foster et al. reported that the age distribution for fibroadenoma ranged from childhood to more than 70, with a mean of about 30 years and a median of about 25 years.

The malignant neoplasms included patients in the age range of 31 to 70 years while maximum number of cases (13 cases) belonged to the age group of 41 to 50 years. This was similar to the findings of Raina et al.[10]who found a similar age distribution in patients of breast carcinoma in north India with the mean age at diagnosis being 47 years. Breast cancer has been reported to occur a decade earlier in Indian women than their western counterparts. The mean age of incidence is about 42 years in India as compared to 53 years in white women [11]

In our study diffuse CD34 expression was seen in the interlobular as well as intralobular stroma of the breast. However, the origin of these stromal cells remains controversial with some researchers advocating that CD34+ fibroblasts are present in the stroma since birth (Barth et al.,2002), while others are of the view that they are derived from blood borne fibrocytes (Catteau et al.,2003). Some investigators have reported that CD34 positive stromal cells disappear in the stroma of invasive ductal carcinoma (IDC) of the breast [2,7,12]. Most of the benign lesions in our study (fibroadenoma, fibrocystic disease, adenosis and benign phyllodes) showed a moderate to dense and diffuse staining for CD34 in the stroma (grade 2+ t0 3+). Similar findings have been demonstrated in previous studies [6,13,14]

The status of CD34 expression in intraductal papilloma has not been studied extensively. Kuroda et al (2004) showed negative to focal staining in cases of papilloma, in contrast to other benign lesions[15]. In our study intraductal papilloma showed less intense staining than other benign lesions.

Overall, the benign lesions in our study showed retained expression of CD34 with a grade of 2+ to 3+.

There was consistent loss of CD34 in all malignant tumors in our study, irrespective of the histologic type. This is similar to the findings of various authors earlier[2,6,13,14]. All the previous authors in this field have shown loss of CD34 in case of breast malignancies. But there are certain disagreement regarding the loss of CD34 in case of IDC and ILC. While Catteau et al. and Cimpean et al., proposed that infiltrative lobular carcinoma shows less marked loss of CD34 as compared to IDC; Kuroda et al., and Khan AA et al., demonstrated similar extent of CD34 loss in both.[14,15]

The reason for loss of CD34 stromal cells in malignancy is unknown. Hence, overall, most of the benign lesions in this study retained CD34 expression in their stroma in contrast to almost complete loss of CD34 positive stromal cells in all invasive carcinomas. There is significant difference in loss of CD34 expression in benign and malignant lesions . Therefore, CD34 can be used to differentiate between benign and malignant lesions of the breast, where retained or diffuse CD34 expression in the stroma would suggest a benign lesion whereas loss of stromal CD34 would indicate malignancy.

V. Conclusion

Besides being expressed in the wall of blood vessels, there is diffuse expression of CD34 in the normal breast stroma. Its expression is retained in most of the benign breast lesions. The expression of CD34 in stroma of malignant lesions is almost completely lost in all types of malignant neoplasms. Due to the significant differences in CD34 expression between benign and malignant breast lesions, it can potentially be used to

differentiate between the two and can be used as an important diagnostic marker. In future, further studies can also be done to establish its role as therapeutic target in cases of breast cancer.

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Angel Tete, et. al. "A study of CD34 antigen expression in benign and malignant breast lesions in patients attending RIMS, Ranchi." *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*, 21(02), 2022, pp. 37-43.