A CROSS-SECTIONAL STUDY OF COX-2 (CYCLO-OXYGENASE 2) IMMUNOHISTOCHEMICAL EXPRESSION IN BREAST CARCINOMA AT A TERTIARY CARE TEACHING HOSPITAL IN CHENGELPET DISTRICT

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Abstract: The breast carcinoma represents one of the most common malignancies among women and has been a cause of death among cancer victims in the world. Learning how to identify dependable prognostic biomarkers is important in enhancing the evaluation and management planning of diseases. The aim of the study was to measure the level of the immunohistochemical expression of Cyclooxygenase-2 (COX-2) in breast carcinoma and establish its correlation with clinicopathological parameters. On 64 histopathologically confirmed cases of breast carcinoma, a cross sectional observational study was done. Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections with the COX-2 and staining intensity was graded using a semi-quantitative scoring system. Spearman rank correlation was used as a measure of statistical analysis. COX-2 was positive in three-quarters of the cases, and high levels were found in 31.3% of tumours. COX-2 expression levels exhibited significant positive correlations with the tumour size, histological grade and pathological stage, revealing that the higher the COX-2 expression is, the more aggressive any of the described tumour characteristics are expected to be. Nevertheless, there was a lack of statistically significant correlation between COX-2 expression and the age of patients or their invasion of the lymph node. The research findings indicate that the COX2 overexpression is prevalent in breast carcinoma and it is also linked with poor pathological characteristics. Consequently, COX-2 can be an important prognostic biomarker, and can be a therapeutic target, which further justifies it in the pathological testing of a breast cancer patient to enable the provision of a better prognostic assessment and personalized patient therapeutic intervention.

Keywords: Breast carcinoma; Cyclooxygenase-2 (COX-2); Immunohistochemistry; Prognostic biomarker; Clinicopathological characteristics.

I. INTRODUCTION

Breast carcinoma has been the predominantly diagnosed cancer in women in both developed and developing countries globally and is currently ranked among the most common causes of cancer-related deaths even though there have been major improvements in screening, diagnosis and treatment [1]. The growing worldwide incidence of breast cancer, especially among developing nations, underscores the necessity of quality prognostic biomarkers that can enhance the level of risk stratification, as well as assist personalized treatment plans. Even though traditional prognostic variables, such as tumour size, histological grade, lymph node invasion, and pathological stage are still used in clinical treatment, the vast amount of heterogeneity in disease progression and response to treatment requires the discovery of more molecular markers [2]. Cyclooxygenase-2 (COX-2) is an inducible enzyme that catalyses the transformation of arachidonic acid into prostaglandins that control inflammation, cell growth, apoptosis, angiogenesis and tumour growth. COX-2 in contrast to constitutively expressed COX-1 isoform is upregulated by inflammatory cytokines, growth factors and oncogenic stimuli. There is growing evidence that COX-2 over-expression plays a role in breast carcinogenesis by supporting tumour cell growth, angiogenesis, anti-apoptotic effects, epithelial-to-mesenchymal transitions, and altering the tumour microenvironment [3]. This has led to increased interest in COX-2 as a prognostic biomarker and treatment option in breast cancer. Nonetheless, the existence of published research studies on the prognostic value of COX-2 expression in breast carcinoma has given contradicting results, especially because of differences in the study sample, methods, immunohistochemical scoring, and tumour characteristics. Consequently, additional testing of the association between the COX-2 expression and traditional clinicopathological measures is justified. This current cross-sectional work was performed to evaluate the immunohistochemical expression of COX-2 in histopathologically verified breast carcinoma specimens and understand its relationship with tumour size and histological grade, pathological and lymph node status and age of the patient. The development of such correlations could also help to generate more evidence about the prognostic power of COX-2 and could be used to enhance clinical decision-making and the design of focused treatment strategies to address breast carcinoma.

II. RELATED WORKS

Breast cancer is still among the most common causes of cancer-related morbidity and mortality in women all around the world, and ongoing research focuses on the development of prognostic biomarkers that could enhance the stratification and treatment decisions. Historical prognostic variables, such as tumour size, histological grade, pathological stage, lymph node status, and molecular subtype are still significant in clinical practice, but more focus has been on inflammatory biomarkers, including Cyclooxygenase-2 (COX-2) due to their roles in tumour initiation, progression, angiogenesis and metastasis [15,16]. COX-2 is an inducible enzyme that controls the production of prostaglandins on an inflammatory response and has been associated to be over-expressed in various malignancies including breast carcinoma. Among the first studies, which were carried out by Ristimaki et al., it was revealed that high expression of COX-2 was correlated with bad prognosis and poor survival in breast cancer patients, and hence, COX-2 could be an independent prognostic biomarker [17]. On the same note, Davies et al. also found that there is a strong correlation between angiogenesis and COX-2 expression which implies that COX-2 promotes tumour vascularization by increasing the production of pro-angiogenic mediators, which in turn promote tumour growth and progression [18]. Recent research findings have been found to support the prognostic value of COX-2 in breast carcinoma. Al-Maghrabi and Khabaz found that elevated COX-2 immunohistochemical expression was considerably linked to unfavourable clinicopathological characteristics, such as bigger tumour size, increased histological grade, greater disease stage and poor prognosis rate in patients [19]. Similar results were experienced by Nassar et al. who indicated that augmented manifestation of COX-2 was often detected in invasive breast malignancy and showed positive associations with tumour aggressiveness and severity of the disease [20]. Similarly, Freitas-Alves et al. found that patients with high COX-2 expression had worse survival rates highlighting the fact that it is an effective clinically relevant prognostic marker [21].

A number of researchers have particularly studied the association of COX-2 expression with histopathology features. COX-2 was found to be also a valuable prognostic marker since its overexpression was strongly correlated with high tumour grades and levels of pathological progression, indicating that it may also be relevant to disease progress [22]. Analogously, Maulidan et al. found out a strong positive relationship between the expression of the COX-2 and histopathological grading in patients with locally advanced breast cancer, indicating that tumour dedifferentiation was associated with a rise in the enzyme expression [23]. All these suggest that the expression of COX-2 is likely to increase with increased tumour aggressiveness. The biological processes behind these observations have been investigated as well. Costa et al. showed that the overexpression of COX-2 was significantly correlated with the process of angiogenesis and lymph node metastasis, and therefore COX-2 might facilitate tumour spread with vascular remodelling and metastatic proliferation [24]. Additionally, Ranger

et al. found out that high levels of COX-2 expression were associated with far-off metastasis and this fact was another piece of evidence to the fact that inflammatory signalling processes were contributory to the advanced development of breast cancer [25].

Although the evidence on the prognostic value of COX-2 overall has been consistent, other findings have shown some conflicting results. The authors concluded that despite the common expression of COX-2 in invasive carcinoma in the breast, the correlation between the progression of the disease and COX-2 was weak indicating that COX-2 might have a more major role to play at the initial stages of tumour formation and not at the later stages of cancer development [26]. These discrepancies can be related to variations in the patient populations, sample sizes, methodologies of immunohistochemical scoring, molecular subtypes, and study methodologies. Therefore, additional research is also due to elucidate the prognostic quality of COX-2 expression in various clinical contexts and patients.

III. METHODOLOGY

3.1 Study Design

The type of design used in this research is the cross-sectional study design based on observational study as the goal here is to determine the immunohistochemical expression of Cyclooxygenase-2 (COX-2) in breast carcinoma tissues and also to determine its correlation with several clinicopathological parameters chosen [4]. The cross-sectional design was found to be suitable as it allowed measuring both the expression of COX-2 and the pathology of resected carcinomas of the breast without longitudinal follow-up. The main theme of the study was to ascertain the incidence and degree of COX-2 expression and its association with already known prognostic factors, one of which is the tumour size, histological grade, pathological stage, lymph node status and age of the patient. Archived formalin-fixed paraffin-embedded (FFPE) tissue blocks and the corresponding histopathology records in the Department of Pathology were used to conduct the investigation [5].

3.2 Study Setting

The study was undertaken in the Department of Pathology, Histopathology and Immunohistochemistry Laboratory, Shri Sathya Sai Medical College and Research Institute, Chengalpattu District, Tamil Nadu, India. The hospital is an urban teaching hospital that offers tertiary care to both urban and rural patients with breast cancer. Histopathological processing, microscopic methods and immunohistochemical staining were done in the departmental laboratory using standard laboratory protocols [6]. Twelve months of research spanning to 18 months (1 year and six months) were researched to find eligible breast carcinoma cases that were diagnosed using modified radical mastectomy to screen the eligible cases to be analyzed.

3.3 Study Population and Sample Size

The target population was the women patients with histopathologically-confirmed breast carcinoma who had surgical treatment within the study period. The primary source of pathological material was the paraffin-embedded tissue blocks derived out of the mastectomy specimens which were subjected to immunohistochemical studies.

To obtain the sample size, a standard formula that is used to estimate a single population proportion was used through a reported prevalence of breast cancer of 13.5 in previous research. With the addition of a 10 percent margin to allow potential non-response or cases that were not completely gathered, the minimum needed sample was established to be 64 cases of breast carcinoma that all met the eligibility assumption and were put in the final analysis [7].

Table 1. Summary of Study Design

Component	Description
Study design	Cross-sectional observational study
Study centre	Department of Pathology, Shri Sathya Sai Medical College and Research Institute
Study period	18 months

Study population	Female patients with histopathologically confirmed breast carcinoma
Sample size	64 cases
Sampling technique	Consecutive sampling of eligible archived cases
Tissue source	Formalin-fixed paraffin-embedded mastectomy specimens
Statistical software	SPSS Version 16.0

3.4 Eligibility Criteria

The cases were only limited to histopathologically confirmed invasive forms of breast carcinoma and the patient must be female. The study population was composed of patients who underwent modified radical mastectomy, or mastectomy and whose archived tissue blocks could be analyzed using immunohistochemical studies. Invasive ductal carcinoma (no special type) and invasive lobular carcinoma were mainly the histological subtypes [8].

They eliminated cases that were benign breast lesions, mesenchymal tumours, myoepithelial tumours, male breast carcinoma and those patients who had undergone neoadjuvant chemotherapy prior to surgery. Likewise, there were cases that are solely represented by biopsy samples without a precise surgical excision since comprehensive pathology could not have been conducted [9].

3.5 Data Collection Procedure

The pathology request forms and hospital records along with the histopathology report retrieved clinical and pathological information. The demographic variables were age of the patient and the pathological variables were tumour size, histological subtype, histological grade, lymph node involvement, tumour stage, surgical procedure. The radiological results of mammography, ultrasonography and computed tomography were also examined where possible to enhance the pathological data [10].

The chosen mastectomy samples had been standardized in 10% neutral buffered formalin, then processed routinely. A total of representative tissue sections were placed in paraffin blocks, and following a rotary microtome, the tissue sections (3-4 0.1mm thick) were stained with haematoxylin and eosin, followed by histopathological examination. The classification of tumours was based on the World Health Organization classification of breast tumours. Histological grading was done based on the Nottingham (Elston-Ellis modification of the Scarff-Bloom-Richardson) grading system and pathological grading was done on the basis of American Joint Committee on Cancer (AJCC)- grading system, eighth edition of the TNM [11]. All the pathological reports were made according to the College of American Pathologists (CAP) recommendations.

3.6 Immunohistochemical Analysis

The paraffin blocks were cut into other sections of around 4 μm thickness to be subjected to immunohistochemical staining. Cyclooxygenase-2 immunostaining in the sample was done using a rabbit monoclonal antibody against Cyclooxygenase-2 (Thermo Scientific, USA) dilution of 1:100. The streptavidinbiotin amplification system was used in line with the instructions provided by the manufacturer.

Colorectal carcinoma tissue was used to give positive control due to known expression of COX-2 and some negative control slides were made by exclusion of the primary antibody. The staining was considered positive only when it was observed in the cytoplasm of tumour cells. Light microscopy was used to examine all stained slides without any reviewers so as to reduce bias on the part of the observers and to have uniform consistency in the interpretation [12]. The use of positive and negative controls during the process of staining was part of quality assurance procedures.

3.7 Assessment of COX-2 Expression

The expression of COX-2 was measured semi-quantitatively by measuring both the intensity of staining, and the percentage of positive stained tumour cells. The intensity of staining was classified in four levels:

- 0+: No staining
- 1+: Weak staining
- 2+: Moderate staining
- 3+: Strong staining

The score of the final product of immunohistochemical using percentage and intensity scores was from 0 to 6. Scores that had scores ranging 0 to 2, were categorized as low or negative expressions and scores 3 to 6 were regarded as positive or high expressions. This standardized method of scoring allowed the objective comparison of the COX-2 expression of the various clinicopathological variables.

Table 2. Variables Included in the Study

Variable	Measurement/Classification
Age	Years at diagnosis
Tumour size	Maximum diameter (cm)
Histological type	WHO classification
Histological grade	Nottingham grading system (Grade I–III)
Pathological stage	AJCC TNM (8th edition)
Lymph node status	N0, N1, N2
COX-2 expression	Immunohistochemistry (0+, 1+, 2+, 3+)
Final COX-2 status	Low (0–2) or High (3–6)

3.8 Statistical Analysis

All the data obtained were inputted into Microsoft Excel and then exported to the statistical package of social sciences (SPSS Version 16.0) so as to be analyzed statistically. Means and standard deviations were used to summarize continuous variables like age and tumour size, and frequencies and percentages to describe categorical variables, which included histological grade, pathological stage, tumour subtype, lymph node status and COX-2 expression.

Since the COX-2 expression was measured with an ordinal scale, and a number of the clinicopathological variables were non-normally distributed, Spearman rank correlation coefficient was employed to test relationships between COX-2 expression and patient age, tumour size, histological grade, pathological stage and involvement of the lymph node. Correlation coefficients and the corresponding p-values were obtained and p-value above 0.05 was considered as being statistically non-significant which was defined as statistical significance [13].

3.9 Ethical Considerations

The study was commenced with ethical approval to conduct the study by the Institutional Ethics Committee. The study was in line with the Indian Council of Medical Research National Ethical Guidelines on Biomedical and Health Research Involving Human Participants (2018). Tissue samples that were archived were utilized with no further invasive procedures being done on the samples [14]. Anonymity of patient records was ensured by using unique identification codes and only certified investigators were given access to research data, and all data on

electronic pipelines was stored in systems that used passwords. Involvement was voluntary, informed consent obtained where necessary, and generalized all findings shown to protect anonymity of individual patients.

IV. RESULTS AND ANALYSIS

The study involved 64 histopathologically validated cancer incidences of the breast. The patients average age was 50.50 and SD of 9.56 years with current tumour mean of 5.48 and SD of 2.47 cm which held that most of the patients were presented to the office in the middle-age, and that their tumours were relatively large at the time of diagnosis [27]. Morphological analysis revealed that invasive ductal carcinoma was the most common subtype (98.4 of all cases), whereas papillary carcinoma was only 1.6. In respect of tumour differentiation, the highest proportion (39.1%), Grade III carcinoma, was found, and Grade I (32.8%), Grade II (28.1%). The majority of the patients were found to have pathological Stage IIB (45.3%) and then Stage IIA (29.7%), Stage IIIA (21.9%) and Stage IIIB (3.1). These results indicate a significant percentage of patients had a moderately advanced disease.

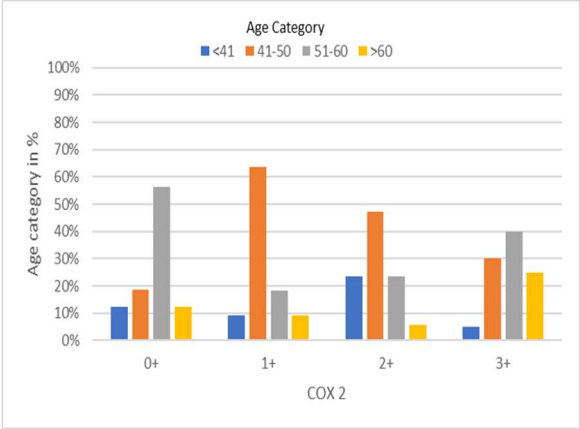

Figure 1: “COX-2 expression in relation to age categories”

Table 1. Demographic and Histopathological Characteristics of the Study Population (n = 64)

Variable	Findings
Mean age (years)	50.50 ± 9.56
Mean tumour size (cm)	5.48 ± 2.47
Invasive ductal carcinoma	63 (98.4%)
Papillary carcinoma	1 (1.6%)
Grade I	21 (32.8%)
Grade II	18 (28.1%)
Grade III	25 (39.1%)
Stage IIA	19 (29.7%)

Stage IIB	29 (45.3%)
Stage IIIA	14 (21.9%)
Stage IIIB	2 (3.1%)

The immunohistochemical analysis showed fluctuating COX-2 levels in cases of breast carcinoma. In general, the proportion of tumours with positive expression of COX-2 was 75 per cent and the percentage with negative expression was 25 per cent. The highest amount of staining (3+) was 31.3 percent, moderate staining (2+) was 26.6 percent, and weak staining (1+) was 17.2 percent. The orientation towards moderate-strong immunoreactivity indicates that overexpression of COX-2 is a prevalent typical molecular character of breast carcinoma and this implies that it could have prognostic significance [28].

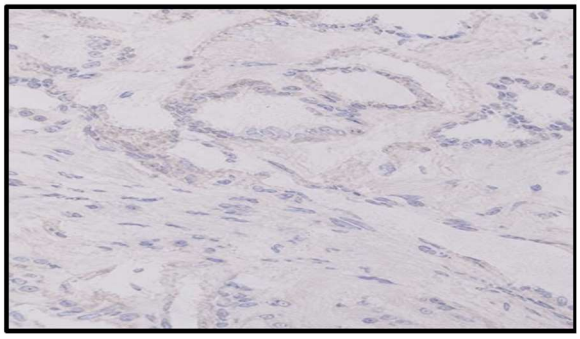

Figure 2: **Immunohistochemical marker COX-2 showing Weak Cytoplasmic positive staining [Score 1+] in Invasive ductal carcinoma NOS [DAB 40X]**

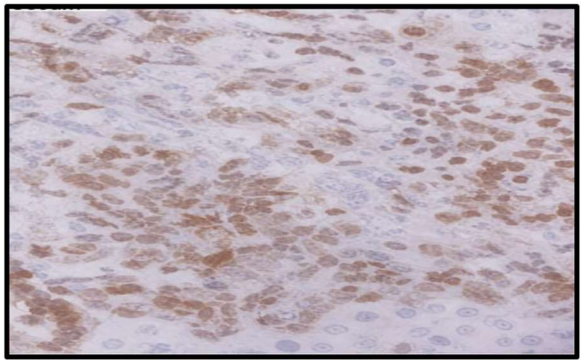

Figure 3: **Immunohistochemical marker COX -2 showing Moderate Cytoplasmic positive staining [Score 2+] in Invasive ductal carcinoma NOS [DAB 40X]**

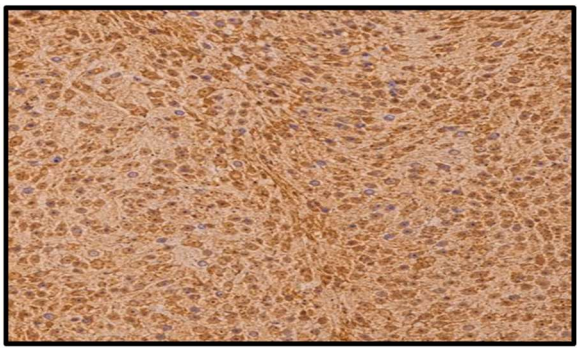

Figure 4: **Immunohistochemical marker COX-2 showing Strong Cytoplasmic positive staining [Score 3+] in Invasive ductal carcinoma NOS [DAB 10X]**

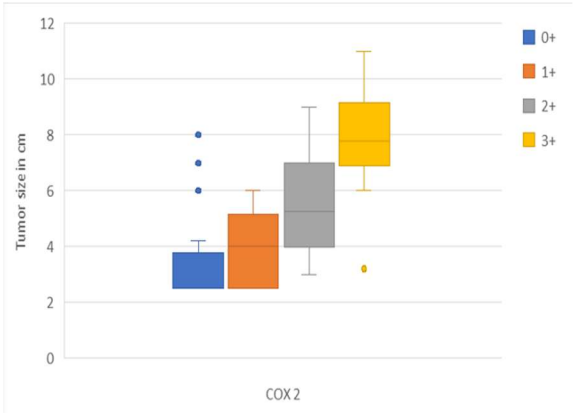

Figure 5: "Relationship between COX -2 expression and tumor size"

Table 2. Distribution of COX-2 Immunohistochemical Expression

COX-2 Expression	**Frequency (%)**
0+ (Negative)	16 (25.0)
1+ (Weak)	11 (17.2)
2+ (Moderate)	17 (26.6)
3+ (Strong)	20 (31.3)
Positive (1+–3+)	**48 (75.0)**
Total	**64 (100)**

Spearman rank correlation was used to examine the relationship between COX-2 expression and patient age. The statistically significant association was not found (0.072, p = 0.574). Samples with patient groups of all ages showed both a different expression of COX-2 and no gradual increase in the intensity of the staining over

time with increasing age. COX-2-negative cases were mostly found in women with ages of 51-60 years, and positive staining was scattered throughout the age brackets. The results indicate that the age of the patients did not serve as a determinant on its own of COX-2 expression in breast carcinoma.

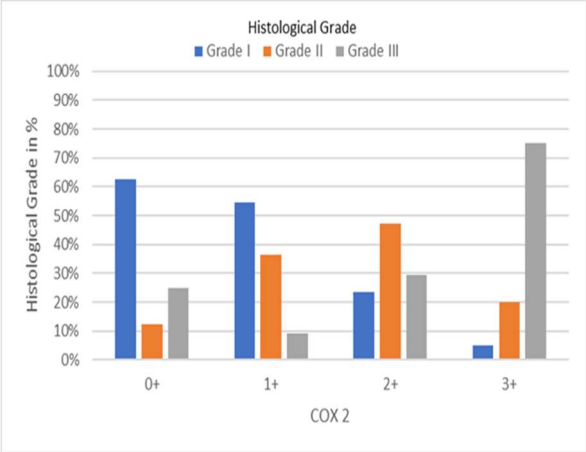

Figure 6: "COX-2 expression in relation to histological grade"

Table 3. Association Between COX-2 Expression and Age

Variable	Statistical Test	Correlation (ρ)	p-value	Interpretation
Age vs COX-2 expression	Spearman correlation	0.072	0.574	Not significant

The examination of tumour size revealed that there was a positive correlation between tumour size and COX-2 expression. The increasing trends of mean tumour size correlated with staining intensity, and ranged between 3.57 ± 1.80 cm in COX-2-negative tumours and 7.89 ± 1.81 cm in strongly positive tumours. The correlation indicated that there was a very significant positive relationship between the two variables (Spearman 0.729, $p < 0.001$). These results demonstrate that a high level of COX-2 expression is strongly correlated with larger tumour size, and so COX-2 might play a role in tumour growth and subsequent disease development in breast carcinoma [29].

Table 4. Association Between COX-2 Expression and Tumour Size

COX-2 Score	Mean Tumour Size (cm)
0+	3.57 ± 1.80
1+	3.91 ± 1.40
2+	5.43 ± 1.77
3+	7.89 ± 1.81
Spearman's ρ	**0.729**
p-value	**<0.001**

The correlation between the COX-2 expression and the histological grade revealed that the association between them is statistically significant and positive (Spearman 0.525, p = 0.001). COX-2- negatives were Grades I (62.5%), strong COX-2 expression (63 plus) was noticed in Grade III (75%). The moderate expression was spread out among all three grades and weak expression was more commonly observed in Grade I and Grade II lesions. These results suggest that the up-regulation of COX-2 is linked with a decreased differentiation of tumours and therefore COX-2 can be used as a marker of violent tumour biology. On the same note, the pathological stage was also significantly positively correlated with COX-2 expression (Spearman 0.391 p < 0.01). The greatest prevalence of Strong COX-2 occurred in Stage IIB and Stage III disease with the lowest prevalence of COX-2-negativity occurring in Stage IIA. The gradual increase in staining levels with the increase in pathological stage favour the contribution of COX-2 in tumour development and severity of the disease [30].

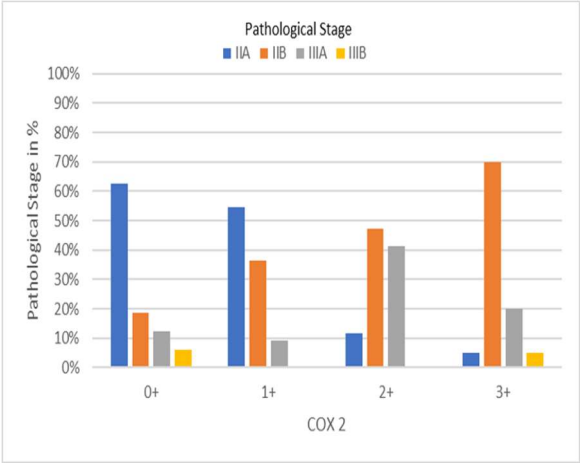

Figure 7: “COX-2 expression across pathological stages”

Table 5. Association Between COX-2 Expression and Clinicopathological Parameters

Clinicopathological Parameter	Spearman's ρ	p-value	Interpretation
Histological grade	0.525	0.001	Significant positive association
Pathological stage	0.391	<0.01	Significant positive association
Lymph node invasion	0.122	0.335	No significant association

The correlation between COX-2 expression and lymph node invasion was tested as well. Even though a case with moderate and strong COX-2 expression had relatively higher values of nodal involvement than COX-2-negative tumours, the general correlation was not found to be significant at a 335.335 p-spearman value (Spearman 0.122). A majority of all the cases in all groups of expression continued to be rated as N0, which means that there is no metastatic involvement of the lymph nodes. Thus, the current results indicate that in this study population COX-2 expression was not significantly linked with lymph node status. Generally, the immunohistochemical test revealed that COX-2 was present in three-quarters of the breast cancers and showed a strong correlation with the tumour size, histological grade, and pathologic stage. The highest COX-2 staining was always found in larger tumours, poorly differentiated carcinomas, and higher pathological stages, implying that progression of disease is consistent with augmented COX-2 of the organism. On the other hand, there was no statistically significant correlation found between the COX-2 expression and patient age or lymph node invasion. Taken together, these results indicate the possible usefulness of COX-2 as a prognostic biomarker in breast carcinoma and its overexpression as indicative of progressively greater tumour aggressiveness and not just of demographic factors or regional nodal metastasis.

V. CONCLUSION

This study has assessed the level of Cyclooxygenase-2 (COX-2) immunohistochemical expression in breast carcinoma and how this is linked to the known clinicopathological facts. The results indicated that COX-2 was positively expressed in most of the breast carcinoma implying that over-expression of this inflammatory enzyme is a typical characteristic of the ailment. The statistical result showed that there were positive significant relationships between COX-2 expression and tumour size, histological grade, and pathological stage, indicating the more the COX-2 is expressed, the more the aggressiveness of the tumour, and the progression stage of the disease. Conversely, the expression of COX-2 did not show a statistically significant correlation with age of the patients and the lymph node invasion. These findings suggest that the COX-2 is much more strongly associated with the intrinsic tumour behaviour rather than with demographics or the regional nodal involvement. In general, our study indicates that COX-2 can be a promising predictive biomarker in breast carcinoma. The presence of negative pathological characteristics connotes that COX-2 immunohistochemical analysis could be used to supplement existing histopathology analysis because of the information that it offers that could help in prognostics. Moreover, the results support the role of inflammation in the progression of breast cancer and identify COX-2 as a promising therapeutic option. These findings should be replicated in future prospective multicentric studies with larger samples, extended follow-up, and molecular profiling to corroborate the findings and to more definitively determine the value of COX-2 in individualized breast cancer treatment.

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