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Research Protocol

Pathology Section

Utility of COX-2 as Immunohistochemical Prognostic Marker in Relation to Various Histopathological Parameters and TNM Staging in Colorectal Carcinoma

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ABSTRACT

Introduction: The most prevalent neoplasm of the gastrointestinal system is colorectal carcinoma. After lung and breast carcinoma, malignant colorectal cancer appears to be characterised by inflammation. Among the numerous recognised indicators of inflammation, Cyclooxygenase-2 (COX-2) has been identified as playing a key role in the early phases of carcinogenesis. The link between higher expression of COX-2 and the early stages of carcinogenesis and cancer development implies that COX-2 might be a target for pre-cancerous colorectal lesion imaging.

Aim: To assess the expression of COX-2 in colorectal carcinoma and the association with various histopathological parameters and Tumour Node Metastasis (TNM) staging.

Materials and Methods: The current study is an observational, cross-sectional, retrospective, and prospective study that will take place in the Histopathology and Immunohistochemistry unit of the Department of Pathology, Jawaharlal Nehru Medical College, Sawangi (Meghe), Wardha, Maharashtra, India. Sample collection will be done during the the period of august 2021 to july 2024. The study included approximately 60-65 resected specimens from confirmed and planned Colectomy, Hemicolectomy, and Proctocolectomy specimens received in the Department of General Pathology, JNMC. Observations and results will be collected from the immunohistochemistry study by using COX-2 as a prognostic marker in colorectal carcinoma and correlating it with the TNM staging. Statistical analysis will be made by chi-square test and regression analysis.

Keywords: Cyclooxygenase 2, Colectomy specimens, Tumour node metastasis staging

INTRODUCTION

The most prevalent neoplasm of the gastrointestinal system is colorectal carcinoma. After lung and breast carcinoma, it is the world's third most common cancer and the second leading cause of death [1]. It contributes to ten percent of worldwide cancer incidence and deaths. Epidemiological studies show colorectal cancer contributes to an estimated 1.9 million incidence cases and 0.9 million mortalities globally in 2020 [1]. In comparison to Western countries, the incidence rate is low in India. The incidence of colorectal carcinoma is relatively less among the younger population (<30 years) than in the elderly population. The majority of Indian research determined that the typical age of incidence for this tumour is 45 to 84 years old, with a male predominance.

Risk factors for colorectal carcinoma can be classified as genetic and environmental or lifestyle-related factors. Colonic polyposis syndromes, Familial Adenomatous Polyposis (FAP) and its variations (Turcot, Gardener, and attenuated FAP), and non polyposis syndromes are inherited illnesses that lead to colorectal cancer (Lynch syndrome) [2]. Environmental risk factors include modifiable and non modifiable variables. Lack of physical exercise, low fibre-high fat diet, alcohol drinking and tobacco use are all controllable risk factors. Non modifiable risk factors include age, gender, and ethnicity. African, Americans population at an increased risk of developing colon cancer than other races, colorectal carcinoma is more common in people who have Crohn's disease or ulcerative colitis [3].

Variety of symptoms, including changes in bowel habits, discomfort,tenesmus, as well as rectal bleeding and surgical emergencies including intestinal obstruction [2]. The clinical symptoms may vary based on the tumour location and tumour staging. Unfortunately, colorectal tumours grow slowly and may go unnoticed for lengthy periods of time [2].

Colorectal carcinoma can be assessed by using various investigations which include complete blood count, liver function test, imaging technique such as X-ray, computed tomography, magnetic resonance imaging, PET scan) [3], diagnostic colonoscopy, proctoscopy,biopsy and evaluation of tumour markers on immunohistochemistry [3].

The prognosis of colorectal adenocarcinomas is related to clinical and pathological parameters such as age, sex, tumour size, the local extent of the tumour, tumour edge, tumour margin, tumour thickness, lymph node involvement and the most important is tumour stage [2]. Prognosis of newly diagnosed colorectal cancer primarily on the stage defined by the TNM, the American Joint Committee on Cancer Classifications, and the CAP guidelines [4].

Immunohistochemistry is the technique of using the antigen-antibody interaction to identify particular antigens on cells. It involves the process of selective identification of antigens, Antibodies that bind selectively to antigens in biological tissues are used to detect a protein in the cells of a piece of tissue [5].

The Cox isoenzymes also known as prostaglandin (PG) rate-limiting synthases catalyse the metabolism of Arachidonic Acid (AA) to PG H2 [6]. Other PGs and thromboxanes are formed from it. Finally thromboxane A2 (TXA2) and physiologically active PGs (PGD2, PGE2, PGF2, and PGI2) are formed [6]. These regulatory chemicals are important in a series of biological processes which include cell proliferation, angiogenesis, immunological function, and inflammation, all of which are important in the formation and progression of cancers [5].

Malignant colorectal cancer appears to be characterised by inflammation. COX-2 has been identified as playing a key role in the early phases of carcinogenesis. The link between higher expression of COX-2 and the early stages of carcinogenesis and cancer development implies that COX-2 might be a target for pre-cancerous

colorectal lesion imaging. As a result, the current study is to assess the expression of COX-2 in colorectal cancer and correlate it with the histopathological grade, nodal status, and TNM staging.

Hence, this research protocol is planned to assess expression of COX-2 in colorectal carcinoma in relation with histopathological parameters and TNM staging. The objective of this study is to confirm and diagnose colorectal carcinoma by histopathological examination and to determine histological grades based on COX-2 as a prognostic markers; The authors will also determine the staging of colorectal carcinoma by TNM classification based on eighth American Joint Committee of Cancer (AJCC). The authors will assess COX-2 expression in tumour tissues of colon and rectum by immunohistochemistry. The research protocol will compare the relation between COX-2 expression with the histopathological parameters, histological grade and pathological TNM staging.

MATERIALS AND METHODS

The planned research protocol will result in cross-sectional, retrospective and prospective study that will take place in the Histopathology and Immunohistochemistry unit of the Department of Pathology, between the years of 2022 and 2024. The approval for the research protocol has been taken from university's Institutional Ethical Committee (DMIMS (DU)/IEC/2022/105).

Sample size determination: Sample size calculation for study on estimating a population prevalence has been described by Daniel ET in study by Charan J and Biswas T:

$n=Z^2p.(1-p)/d^2$

In this formula [8], 'n' is the sample size, 'Z' is the statistic denoting the level of confidence (significance level of 5% i.e., confidence interval of 95%=1.96), 'P' denotes the expected prevalence of colorectal carcinoma (18%) and 'd' is the desired level of error in margin (7%). The sample size (n) is derived to be 63.85 (using above factors).Thus, nearly 60-65 patients will be needed in this study group.

Inclusion criteria: Patients diagnosed with Colorectal Carcinoma (primary cases arising denovo, without any history of previous treatment)andcasesofcolectomy/Hemicolectomy/Proctocolectomy resection specimens are planned to be involved in the study.

Exclusion criteria: All inflammatory lesions and other malignancies of the gastrointestinal tract, biopsy specimens and all treated cases of colorectal carcinoma and cases with recurrence will be excluded from the study.

Planned Procedure

The study will include approximately 60-65 resected specimens from confirmed and planned colectomy, Hemicolectomy, Proctocolectomy specimens received in the Department of Pathology.

Staining Protocol: Haematoxylin and Eosin staining [7]:

The positive tissue control considered will be a piece of lung adenocarcinoma tissue. Colon cancer tissue sections will be de-paraffinised by immersing into three sets of xylene for ten minutes each. It will be followed by three sets of absolute ethanol and finally rinsed with tap water for de-waxing of the sections. Post this, alcohol in descending grades will used to rehydrate the prepared sections which will then be exposed to water. The slides will then placed into Harris Haematoxylin for ten minutes and then rinsed thoroughly in running tap water for approximately four to five minutes. The sections will be exposed to one percent Hydrochloric (HCL) acid in 70% alcohol to remove excess Haematoxylin followed by wash with alkaline tap water for five minutes. The slides will be then stained in Eosin (1 percent aqueous) for one minute. The sections will then be dehydrated by washing with absolute alcohol (90 percent), before being mounted in Dibutylphthalate Polystyrene Xylene (DPX).

Approach to the study: Patients who are enrolled in the trial will be asked to give their prior informed permission so that specimen from clinically suspicious cases would be obtained and forwarded to the Department of Pathology for histopathological analysis. In this study, we will collect colectomy/Hemicolectomy/Proctolectomy specimens which will be preserved in 10 percent formalin in the Department of Pathology, for histopathological examination. We will perform gross inspection and dissection of this surgical specimen then we will take the sections from the margins, tumour mass, and lymphnodes. After that we will perform regular tissue processing followed by Haematoxylin and Eosin (H&E) staining. Then we will assess the tumour's histological grade by categorising on the basis of The College of American Pathologists (CAP) [4] standards. We will analyse tumour's stage by TNM staging based on 8th American Joint Committee on Cancer, (AJCC) [4].

Immunohistochemical staining for COX-2 [6]:

The segment will be cut at nearly four micrometres, then it has to be floated on Poly-L Lysine coated slides, and then incubated at thirty seven degrees celsius for duration of one day and then it has to be shifted to fifty eight degrees celsius overnight. Deparaffinisation will be accomplished in two changes of Xylene for fifteen minutes each, followed by dexylinisation in two changes of pure alcohol. De-alcoholisation will be done for one minute with ninety percent & seventy percent alcohol, respectively. Re-hydrate for ten min in tap water and then for five minutes in water processed by distillation. Retrieval of antigen will be accomplished after ten minutes of pressure cooking in citrate buffer (pH 6.0) and then soaking the pressure cooker in water for twenty minutes in the sink. After that, the slides will be washed for five minutes in distilled water.

For five minutes, the slides will be immersed in Tris-Buffer Solution (TBS) (pH 7.6). Peroxide block will be applied for ten to fifteen minutes. The slides will then be washed three times in TBS buffer, each time for five minutes. A fifteen-minute power block will be used to prevent non specific reactions with the other tissue antigen The slides will be drained, and the sections will be coated with the appropriate primary antibody for an hour to identify tumour markers using an antigen-antibody response. The slides will then be washed three times in TBS for five minutes each time to remove any unbound antibodies. A final super-enhancer will be added and allowed for thirty minutes to improve the response between the primary and secondary antibodies. The unbound antibodies will be removed by washing under TBS buffer. The surplus stain will be rinsed in tap water for five minutes after Counter staining using a haematoxylin stain for one minute. The slide was then air-dried, xylene cleaned, and DPX mounted.

The positive control will be a piece of lung adenocarcinoma, while the negative control will be the same tissue treated without secondary antibody 70. 3'3' diaminobenzidine will be the chromogen in the colour development solution (DAB).

Methodology of interpretation [5]:

The extent of staining and the intensity of staining will be added together for a total score expression of COX-2 will be scored as per the criteria shown in [Table/Fig-1-3].

Interpretation based on histologic grade according to College of American Pathologists guidelines (CAP) [4]:

The criteria for grading considered in the present study is as

Extent of staining (Proportion Score PS)	Percentage of positive cells
0	0%
1	1%-25%
2	26%-50%
3	51%-75%
4	76%-100%

[Table/Fig-1]: Extent of staining scoring.

Intensity of Staining (IS)	Percentage of positive cells
0	Negative
1	Weak*
2	Intermediate**
3	Strong***

[Table/Fig-2]: Intensity of staining scoring.
*Weaker than inflammatory cells
**Same as inflammatory cells
***Stronger than inflammatory cells

Total score (PS+IS)	Interpretation
0-2	Negative
3-4	Low positive
5-7	High positive

[Table/Fig-3]: Interpretation of total score.

follows:

- Grade I- Well-distinguished
- Grade II- Differentiated to a moderate extent
- Grade III- Insufficiently differentiated
- Grade IV- Undifferentiated

Despite high interobserver heterogeneity, multivariate analysis has repeatedly demonstrated that histologic grade is a stage-independent predictive predictor. High tumour grade, in particular, has been shown to be an unfavorable prognostic factor. The number of grades has been compacted in the bulk of research reporting the prognostic value of tumour grade to establish a two-tiered stratification for data analysis as follows:

- Low-grade: Well-differentiated and moderately differentiated
- High-grade: Poorly differentiated and undifferentiated

  The classification of low-grade tumours as well-differentiated or moderately differentiated has been the most significant grading discrepancy faced, whereas interobserver variability in recognising high-grade carcinoma is modest. A two-tiered grading system for colorectal cancer (low-grade and high-grade) is suggested due to its shown predictive efficacy, relative simplicity, and consistency. The following are the criteria for grading only on gland development.
- **Low-grade=** Low-grade gland formation is defined as more than or equal to fifty percent gland formation.
- **High-grade=** Less than fifty percent gland development is considered high-grades.

Observations and results will be collected and combined together over the period of three years and will be analysed statistically.

STATISTICAL ANALYSIS

Statistical analysis will be made by chi-square test (Software Used: Statistical Package for Social Sciences (SPSS) version 27.0) to determine the association between COX-2 expression and histopathological features. Multiple linear regression analysis is performed to determine the relative elements that contribute to metastasis. A p-value of <0.05 is considered to indicate significance.

DISCUSSION

Colorectal carcinoma is the world's third-highest cause of cancer-related deaths in both genders, with an anticipated 515,637 male fatalities and 419,536 female deaths in 2020 [1]. The most abundant prostanoid present in colorectal tissue is COX-2 produced from PGE2. The COX-2 expression is either low or non-existent in the typical colonic mucosa. In the normal mucosa, macrophages, vascular endothelial cells, and neuroendocrine cells produce low amounts of COX-2 [5]. However, it is an early response gene that is rapidly activated by growth factors, cytokines, oncogenes, and phorbol ester. The COX-2 promoter region has multiple transcription factor binding sites, including nuclear factor Kb, nuclear factor of Interleukin (IL)-6, AMP (Cyclic Adenosine Monophosphate), and Hypoxia-Inducible Factors (HIF-1). All of which up-regulate COX-2. COX-2 has been linked to cancers of the colon, breast, bladder, oesophagus, and prostate, as well as precancerous and malignant lesions [5]. Cox-2 contributes towards carcinogenesis by: i) Evasion of apoptosis; ii) Providing self-sufficiency of growth signals; iii) Decreasing the sensitivity to anti-growth signals; iv) Providing boundless potential to replicate; v) Supporting angiogenesis; vi) Progression of the tumour by promoting invasion and metastasis. Avoidance of the anti-tumour immune response. COX-2 is implicated in carcinogen activation and apoptosis suppression, replicative potential, angiogenic factor generation, and metastatic potential enhancement [5].

Steinbach G et al., studied the effects of Celecoxib, a selective COX-2 inhibitor, which has been shown to have beneficial effects on patients with FAP [9]. According to many studies, such patients have a one-hundred percent chance of developing colorectal cancer. In this placebo-controlled research, Colorexib was given to a group of patients and the effect it had on them was monitored for six months. According to the findings of this study, regular ingestion of colorexib can lessen the occurrence of colorectal polyps.

Sano H et al., conducted an immunohistochemical analysis to show the exaggerated appearance of COX-2 in colon tissues with cancer [10]. This study intended to examine the role of Non Steroidal Anti-Inflammatory Drugs (NSAIDs) in reducing the risk of colorectal cancer. The major observations are as follows:

- Immunoreactive COX-2 was found in the mucosal epithelial cells, mucosal and mononuclear cells, Auer Bach's myenteric plexus, vascular endothelial cells, and smooth muscle cells of the inner circular and external longitudinal layers.
- In cancer cells, the extent and intensity of the immunoreactive COX-2 were greater than that of mucosal epithelial cells, inflammatory mononuclear cells, and fibroblasts in colorectal cancer tissues.
- These findings suggest that COX-2 may have a role in the genesis, progression, and maintenance of colorectal malignancies when activated by chemical compounds, cytokines, and growth factors.

The function of COX in endothelial cell migration and angiogenesis has been studied by Fujita T et al., [11]. The researchers developed in-vitro model systems in which endothelial cells and colorectal carcinoma cells were co-cultured. COX-2 overexpression causes cells to create PGs, which encourage endothelial migration and tube formation, whereas control cells used by the researchers showed limited activity. Various angiogenic factors, along with NS-398 (a form of COX-2 inhibitor) and aspirin can inhibit this effect by antibodies. Based on the observations from in-vitro analysis, the team concluded that:

- Cells, where COX-2 is overexpressed, would show better growth as compared to Caco-2 cells.
- Both indomethacin and NS-398 significantly reduced the growth of tumours.

Additionally, a selective COX-2 inhibitor has little effect on the growth of HCT-116 xenografts, whereas a nonselective COX-2 inhibitor causes cancer cells to release angiogenic factors. Kasper HU et al., an immunohistochemical assessment of COX-2 expression in colorectal carcinoma and liver metastases in 57 persons indicated that COX-2 is consistently engaged in the manifestation of metastatic colorectal cancer [12]. The findings of the study indicated that COX-2 inhibitors may have anti-cancer effects through changing signaling pathways linked to cell sensitivity and death.

Al-Maghrabi J according to the study, COX-2 expression was associated to lymph node involvement and distant metastases in 56 percent of patients with colorectal cancer, over expression of COX-2 was also linked to a greater likelihood of tumour recurrence in the study, implying that expression of COX-2 can provide necessary prognostic information in colorectal carcinoma and could be used to screen patients at high risk of recurrence [13]. COX-2-negative patients exhibited a much longer survival time than COX-2-positive patients, indicating that Cyclooxygenase 2 expression is associated to a poor prognosis.

The TNM classification (Tumour/Lymph Node/Metastasis) was devised by the American Joint Committee on Cancer [4] and is the most extensively used clinical staging method for categorising colorectal carcinoma. This method is primarily used for classifying the severity of cancer and helps to assess the various cancer staging. It primarily assesses (T) tumour, regional lymph nodes (N), and distant metastasis (M). Implementing a standard classification system such as TNM helps in knowledge sharing and research efficiently [4].

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