DOI: 10.7860/JCDR/2023/60304.18447

Research Protocol

Pathology Section

Analysis of BRAF V600E in Precancerous and Cancerous Lesions of Colorectum by Immunohistochemistry: A Research Protocol

SHREYA GIRI GOSWAMI[1], ARVIND BHAKE[2], SUNITA VAGHA[3]

ABSTRACT

Introduction: Colorectal cancers have been extensively studied in recent years to understand their molecular abnormalities and their impact on treatment outcomes, as they continue to be a major global health burden. The Adenomatous Polyposis Coli (APC) β-catenin pathway and the Mitogen-activated Protein Kinase (MAPKs) pathway are the topic of extensive research in colorectal pathology.

Need of the study: The mutation in B-type Rapidly Accelerated Fibrosarcoma (BRAF) kinase is known to be associated with the pathogenesis and progression of precancerous lesions to cancerous lesions. Precancerous lesions carry a high risk of developing Adenocarcinoma. Therefore, the expression of BRAF can serve as a useful predictive and prognostic biomarker for disease outcomes and establish correlations with other clinicopathological parameters.

Aim: The present study aims to assess the immunoexpression of BRAF V600E in precancerous and cancerous lesions of the colorectum, as well as the relationship between BRAF expression, histological grading, and the Tumour, Lymph Nodes, and Metastasis (TNM) staging of colorectal cancers.

Materials and Methods: This will be an ambispective observational study (both prospective and retrospective) conducted at the Department of Pathology, Rural Hospital, Wardha, Maharashtra, India, over a two-year period (Retrospective data: August 2021 to July 2022, Prospective data: August 2022 to July 2023). A total of 25 cases each of precancerous and cancerous colorectal lesions will be included. Immunohistochemical expression of BRAF V600E will be performed on tissue sections from each case, and scoring will be conducted. The relationship between BRAF expression and clinicopathological parameters (such as tumour site, tumour size, number of positive lymph nodes, and perineural invasion), histological grade, and TNM stage will be assessed. The results will be analysed using Statistical Package for the Social Sciences (SPSS) software, version 27.0. The Chi-square test will be used to investigate the relationship and association between BRAF V600E expression and clinicopathological parameters. Furthermore, Pearson's correlation coefficient method will be utilised to determine the correlation between two parameters. A significance level of 95% (p-value <0.05) will be considered.

Keywords: Cancer, Immunoexpression, Mutation, Polyp, Prognosis

INTRODUCTION

Cancers of the colorectum have remained a focal area in oncology due to the need to understand the pathogenetic mechanisms, molecular basis of carcinogenesis, and available treatment options. Until a decade ago, colorectal cancers were primarily assessed for their prognosis and predictive outcomes based on histological types, grade, and disease stage. Extensive studies have been conducted on the clinical situations and syndromes associated with precursor lesions, providing insights into the pathogenesis of colorectal cancers. With advancements in molecular genetics, conventional histological typing has been scrutinised, leading to the identification of various molecular pathogenetic mechanisms that can be targeted for colorectal cancer treatment [1].

Colorectal cancer is known to arise from precancerous lesions with a high risk of harbouring cancers such as adenovillous polyps, villous polyps, and other hereditary polyposis conditions. Conversely, hereditary non-polyposis cancers are also observed. The transition from known precancerous to cancerous lesions is well-documented in the literature, highlighting certain molecular defects in the cells [2,3]. Modern-day pathology, with the utilisation of defined molecular techniques, has implicated numerous candidate genes and pathways in the development of colorectal cancer. The APC β-catenin pathway and MAP kinase pathway are among the most important ones [4].

Literature reports studies that focus on the detection of mutations in components of the APC β-catenin pathway, which is a part of the Wingless-related Integration Site (WNT) pathway [5,6]. Precancerous lesions have been found to exhibit Kristen Rat Sarcoma viral oncogene homolog (KRAS) mutations with a prevalence of approximately 10%, whereas cancerous lesions show a higher frequency of such mutations [1]. Loss of function of the Tumour Protein 53 (TP53) is also associated with more aggressive forms of cancer and poorer outcomes in patients with colorectal lesions [1,7]. Microsatellite instability, resulting from DNA mismatch repair deficiency, has also been linked to the development of both precancerous and cancerous colorectal lesions. Molecular changes in the mismatch repair pathway impact cell survival and proliferation in colorectal cancer development. Genes such as MutL Homolog 1 (MHL1), MutS Homolog 2 (MSH2), Transforming Growth Factor Beta Receptor 2 (TGFBR2), Bcl-2-associated X protein (BAX), BRAF, Transcription Factor 4 (TCF4), and Insulin-like Growth Factor 2 Receptor (IGF2R) within the mismatch repair pathway have been extensively reported to be associated with sporadic and familial colorectal cancers [2,8]. Hypermethylation in the absence of mutation, particularly in a subset of microsatellite-stable colon cancers, has also been demonstrated [2,4]. Among the aforementioned molecular defects, BRAF is a single gene defect that has been extensively studied in the past 10 years for both precancerous and cancerous lesions of colorectal cancer [1-5]. However, such studies in the Indian population are scarce.

The BRAF V600E mutation is known to be associated with the pathogenesis of adenocarcinoma of the colon and the progression of colorectal lesions to cancerous ones [9,10]. Studies in the literature have found correlations between BRAF immunexpression and various clinicopathological features, such as familial vs sporadic colonic

cancers, young vs old age, tumour grade, tumour size, nodal stage, TNM stage, and metastasis [11,12]. Some studies have performed BRAF immunexpression to suggest prognosis and treatment options, especially in the form of antibody therapies [12-16].

Based on the available literature, the BRAF mutation status has been recognised for its utility as a prognostic and predictive biomarker. The present protocol is necessary to assist clinicians in planning targeted therapies for colorectal cancers, as there is a limited number of studies on mutant BRAF in combined precancerous and cancerous lesions of the colon in the Indian population. Therefore, the aim of this study is to investigate the immunexpression of BRAF V600E in precancerous and cancerous lesions of the colorectum, as well as the clinicopathological features of colorectal cancers. The following objectives have been identified for the study:

1. To examine the immunexpression of BRAF V600E in precancerous and cancerous lesions of the colorectum.
2. To compare the frequency of immunexpression of BRAF V600E in precancerous and cancerous lesions (adenocarcinoma of the colorectum).
3. To study and correlate the immunexpression of BRAF V600E with the histological grade of colorectal cancer and lymphovascular invasion.
4. To study and correlate the immunexpression of BRAF V600E with the nodal tumour stage of colorectal cancer.
5. To correlate the immunexpression of BRAF V600E with the TNM staging of colorectal cancers.
6. To study and correlate the immunexpression of BRAF V600E with the metastasis of colorectal cancers.

Based on the results of previously published studies on the immunexpression of BRAF in colorectal cancers, it is hypothesised that BRAF plays an important role in the molecular pathogenesis of colorectal cancers and that high BRAF protein expression is associated with disease aggressiveness.

REVIEW OF LITERATURE

Colorectal cancer is one of the most common cancers and a leading cause of death worldwide. Numerous studies have been conducted to assess and correlate BRAF with the molecular mutation status in colorectal cancers.

Kanik P et al., conducted a study on 82 colorectal cancer patients to investigate BRAF and KRAS protein expression using immunohistochemistry on paraffin-embedded tissue blocks and its correlation with various clinicopathological parameters and prognosis [1]. They found that 63% of the tumours were BRAF positive, while KRAS positivity was seen in 34% of the tumours. Patients with positive lymph node status, positive perineural invasion, and preoperative serum Carcinoembryonic Antigen (CEA) levels >5.0 ng/mL showed higher BRAF immunopositivity. No significant correlation was observed between KRAS and clinicopathological parameters. Additionally, a higher positive BRAF expression was observed in advanced-stage patients compared to early-stage patients. However, the study did not find any impact on the survival of colorectal cancer patients. The findings of the study established BRAF as a useful indicator of disease aggressiveness in colorectal cancer.

Shetty O et al., conducted a study to assess the frequency of BRAF mutation patterns in colorectal cancer patients at a tertiary care centre using paraffin-embedded tissue sections [8]. They analysed a total of 298 cases for BRAF gene mutation and found that three cases (1%) showed BRAF V600E mutation, which was associated with metastatic characteristics. The study revealed that the right side of the colon was predominantly affected by BRAF gene mutations. However, the study found a low frequency of BRAF alterations, which had no significant impact on patient survival.

Gonzalez-Colunga KJ et al., conducted a retrospective study on 135 cases of colon cancer [11]. The objective of the study was to evaluate the diagnostic performance of BRAF and its association with histopathological characteristics in colon cancer. Out of 135 cases, 9.6% (13) had BRAF mutations, showing intense and diffuse staining on immunohistochemistry. The study revealed that tumours with BRAF mutations tended to be larger in size. The study concluded that BRAF V600E demonstrated excellent diagnostic capabilities, making it a viable alternative for molecular examinations.

Wasti H et al., conducted a study to establish a correlation between the expression of KRAS and BRAF V600E and the histological grades observed in tissue sections of colorectal cancer [12]. They analysed a total of 51 cases of colorectal cancer, with 72.5% being male and 27.4% being female patients. The majority of the tumours (72.5%) were localised to the left side of the colon. KRAS expression was observed in 80.39% of the cases, whereas 39.2% of the cases showed BRAF V600E expression. The study found no significant correlation between KRAS and BRAF expression. KRAS overexpression showed a positive correlation with histological tumour grades and its variants, while BRAF V600E showed no positive correlation with histological variants and tumour grades. Additionally, the study's findings indicated the presence of BRAF V600E mutation in the normal mucosa surrounding the tumour, suggesting that BRAF mutation may serve as an early event in the development of colorectal cancer. The study highlighted the importance of incorporating BRAF V600E as a standard biomarker in the routine diagnosis of colorectal cancer.

MATERIALS AND METHODS

The study will be conducted as an ambispective observational study, involving both prospective and retrospective data collection. It will be carried out in the Department of Pathology at a rural hospital in Wardha, Maharashtra, India, over a period of two years (retrospective data: August 2021 to July 2022, prospective data: August 2022 to July 2023). Ethical clearance for the study has been obtained with the approval number DMIMS (DU)/IEC/2022/1059. Written consent will be obtained from all subjects participating in the study.

Sample size calculation: The sample size calculation was performed using the incidence rate from a previous study [17]. The formula used was Cochrane's formula:

$$n=(Z\alpha/2)^2 \times p \times (1-p)/E^2,$$

where, $Z^{a/2}$=Level of significance at 5% (95% Confidence interval)=1.96

p=Incidence of colorectal cancer=4.9%=0.049 (GLOBOCON 2020) [17]

E=Error of margin=10%=0.10

$n=1.96^2 \times 0.049 \times (1-0.049)/0.10^2=17.90$

Therefore, the initial sample size of seventeen cases was increased to 25 cases each for the groups of precancerous and cancerous colorectal lesions.

Inclusion criteria: The inclusion criteria for the study are cases diagnosed with precancerous polyps/lesions and adenocarcinoma of the colorectum based on biopsy or surgical specimens.

Exclusion criteria: The exclusion criteria include colorectal biopsies with inflammatory histomorphology and glandular dysplasia, as well as cases with biopsy and blocks that do not have representative histomorphology of precancerous and cancerous lesions. Cases with deficient clinical data and details will also be excluded.

Study Procedure

Methodology and parameters studied: The study will collect patient information, including name, age, gender, registration number, unit,

department, In-Patient/Out-Patient Department (IPD/OPD), as well as clinical findings such as symptoms, examination results, and provisional diagnosis. Basic laboratory work-up, including complete blood count, biochemistry, and tumour marker analysis, will also be conducted using the BRAF V600E marker from Abcam. Radiological investigations, such as abdominal sonography, barium studies, Computed Tomography (CT), and Magnetic Resonance Imaging (MRI), will be noted from the patient's case sheets. The study will collect biopsy and surgically resected specimens, as well as specimens of colectomy for suspected precancerous and cancerous lesions of the colorectum.

Laboratory methods: Grossing of biopsy, polypectomy, and colectomy specimens will be done using different techniques [18]. The sections for the specimens will be processed by an automated histokinette to make paraffin blocks using a microtome. After proper processing, Haematoxylin and Eosin (H&E) staining of paraffin sections will be carried out using standard methods. The histological grading of colorectal adenocarcinoma will be performed based on its degree of differentiation as given by WHO guidelines [19]. Staging of colorectal carcinoma will be done according to the American Joint Committee on Cancer (AJCC) guidelines and Dukes classification [Table/Fig-1,2] [20,21].

Stage	T	N	M
I	T1	N0	M0
	T2	N0	M0
IIA	T3	N0	M0
IIB	T4a	N0	M0
IIC	T4b	N0	M0
IIIA	T1-T2	N1/N1c	M0
	T1	N2a	M0
IIIB	T3, T4a	N1	M0
	T2, T3	N2a	M0
	T1, T2	N2b	M0
IIIC	T4a	N2a	M0
	T3, T4a	N2b	M0
	T4b	N1, N2	M0
IVA	Any T	Any N	M1a
IVB	Any T	Any N	M1b

[Table/Fig-1]: Colorectal Cancer Staging Systems: American Joint Committee on Cancer (AJCC) stage [20].
*T: Tumour; N: Lymph nodes; M: Metastasis

Stage	Features	5-year survival
A	Tumour confined to the submucosa	90-95%
B1	Tumour growth into muscularis propria, no nodal metastasis	90%
B2	Tumour growth through serosa, no nodal metastasis	75%
C1	Tumour spreading to 1-4 regional lymph nodes	65%
C2	Tumour spreading to >4 regional lymph nodes	42%
D	Distant metastasis to liver, lung, bone etc.	>5%

[Table/Fig-2]: Duke's staging of colorectal carcinoma [21].

Immunohistochemical assessment: The paraffin-embedded sections of biopsy and surgically resected specimens diagnosed as precancerous or cancerous lesions of the colorectum (adenocarcinoma) will be selected for immunohistochemistry. Only representative blocks of the lesion will be selected for immunohistochemistry. Immunohistochemistry will be carried out using the biotin-avidin peroxidase complex method with monoclonal antibodies against BRAF V600E [22]. The sections will be rinsed in Tris-Buffered Saline (TBS) and then incubated in a 10% casein solution for 10 minutes. They will be further incubated with a properly diluted primary antibody for 60 minutes. After washing with TBS, the sections will be incubated with an optimally biotinylated secondary antibody for 30 minutes. Following another wash with TBS, the sections will be incubated with freshly prepared labeled streptavidin or streptavidin biotin complex for 30 minutes. After another wash with TBS, the sections will be exposed to a DAB substrate solution. They will then be washed in running water, counterstained with haematoxylin, dehydrated, cleared, and mounted [22]. Scoring of immunoexpression of BRAF V600E in cases of colorectal cancer will be done based on a semi-quantitative scoring [Table/Fig-3] system [13].

Score	Cytoplasmic staining intensity	Proportion
0	Negative	0-10%
1	Weak	11-25%
2	Moderate	26-50%
3	Strong	51-75%
4	Very strong	>75%

[Table/Fig-3]: Cytoplasmic staining intensity will be scored as mentioned [13].

Primary outcome: The expected primary outcome of the study will be the assessment of immunopositivity of BRAF in precancerous and cancerous lesions of the colorectum.

Secondary outcome: The secondary outcome, through its results, will help determine the frequency of immunoexpression of BRAF and its correlation with histological grade, lymphovascular invasion, and TNM staging of colorectal cancer. It may also provide information on the relationship between BRAF immunoexpression and metastatic colorectal cancer and nodal stage.

STATISTICAL ANALYSIS

The software used will be SPSS software, version 27.0. The statistical analysis of the association and relationship between the immunoexpression of BRAF V600E and clinicopathological parameters will be conducted using the chi-square test. The correlation between two parameters will be calculated using Pearson's correlation coefficient method. A significance level or confidence interval of 95% (p-value <0.05) will be considered statistically significant.

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PARTICULARS OF CONTRIBUTORS:
1. Junior Resident, Department of Pathology, Jawaharlal Nehru Medical College, DMIMS (DU), Wardha, Maharashtra, India.
2. Professor and Director, Department of Pathology, Jawaharlal Nehru Medical College, DMIMS (DU), Wardha, Maharashtra, India.
3. Head, Department of Pathology, Jawaharlal Nehru Medical College, DMIMS (DU), Wardha, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Shreya Giri Goswami,
Department of Pathology, Datta Meghe Institute of Medical Sciences, DMIMS (DU), Wardha-442004, Maharashtra, India.
E-mail: shreya18g@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Oct 01, 2022
- Manual Googling: May 17, 2023
- iThenticate Software: Jun 10, 2023 (8%)

ETYMOLOGY: Author Origin

EMENDATIONS: 8

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Sep 20, 2022**
Date of Peer Review: **Nov 26, 2022**
Date of Acceptance: **Jun 14, 2023**
Date of Publishing: **Oct 01, 2023**