Original Article

Expression of BRAF V600E in Tissue Samples of Colorectal Carcinoma and Its Correlation with Various Clinico-Pathological Parameters

Hina Wasti, Summayyah Shawana, Beenish Hussain Nomani, Santosh Kumar Sidhwani, Rubbab Mir, Hareem Fatima

ABSTRACT

Objective: To determine the expression of BRAF V600E in tissue samples of colorectal carcinoma and to correlate it with various clinico-pathological parameters.

Study design and setting: Cross-sectional study was conducted at department of Pathology, Pakistan Navy Station Shifa hospital Karachi from 1st March 2016 to 28th February 2019

Methodology: Total of 51 cases of colorectal cancer were analyzed for immunohistochemical staining using BRAF antibodies on representative tissue blocks. Clinical and pathological records were retrieved for data collection. The results of immunohistochemical analysis were correlated with the recorded clinico-pathological parameters.

Results: In this study 51 cases of colorectal cancer were analyzed for immune expression of BRAF V600E. The age of the patients ranged from 14 to 85 years with the mean age of 60.96 years. Among the 51 cases, 37(72.5%) cases were males and 14(27.4%) were females. 37(72.5%) were localized to left side colon and 14(27.4%) were found in the right colon. For BRAF V600E, positive expression was seen in 20(39.2%) cases, whereas 31(60.7%) cases showed negative expression of BRAFV600E. No significant association was seen between BRAF V600E expression and histological variants like age, gender, tumor location and glandular carcinomas.

Conclusion: BRAF V600E immunosuppression was seen in 39.2% of colorectal carcinoma in this study. No significant association was seen in BRAF V600E expression and histological variants.

Key Words: BRAF V600E Immunohistochemistry, Colorectal cancer, Clinicopathological parameters.

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INTRODUCTION:

Colorectal cancer (CRC) has been identified as the most common cancer of the digestive tract. Being the third most prevalent cancer in both genders. It represents almost 10% of all registered malignant diseases.[1,2] Estimated incidence levels in males for colorectal cancer is significantly greater than in females in major areas of the globe. Recently, a large number of developing countries have shown an acute increase in the incidence of colorectal cancer.[3] In Pakistan CRC accounts for 52% of all gastrointestinal tumors in comparison to other countries.[4]

Colorectal cancer (CRC) is a heterogeneous disease which emerges through several important pathways. Both environmental and genetic factors are responsible for the development of the pathogenesis.[5] However there is a continuous rise of colorectal carcinoma in those under the age of 50.[6] Several genetic and epigenetic mutations have been identified in various proto oncogenes and tumor suppressor genes which involve distinct pathways like, chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylation phenotype (CIMP).[7, 8]

The most commonly occurring mutation in colorectal carcinoma is gain in the function of BRAF proto-oncogene, which act as potent carcinogens in initiation and progression of colorectal carcinoma and plays a significant role in its pathogenesis. BRAF belongs to RAF family of protein and its gene is located on chromosome 7, encoding a 766-amino acid serine/threonine kinase.[9] The vast majority of mutated BRAF is V600E resulting from a point mutation having 80% cancerous potential. This results in constitutive activation

Hina Wasti
Senior Lecturer, Department of Pathology,
Bahria University Medical and Dental College, Karachi
Email: drhinafaisal@gmail.com

Summayyah Shawana
Associate Professor, Department of Pathology,
Bahria University Medical and Dental College, Karachi

Beenish Hussain Nomani
Senior Lecturer, Department of Pathology,
Bahria University Medical and Dental College, Karachi

Santosh Kumar Sidhwani
Assistant Professor, Department of Pathology,
Ziauddin University, Karachi

Rubbab Mir
MCPS Graduate Trainee, Department of Gynaecology,
Jinnah Medical College Hospital, Karachi

Hareem Fatima
Senior Lecturer, Department of Pathology,
Bahria University Medical and Dental College, Karachi

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of RAS-RAF-MAPK pathway. BRAFV600E significantly increases the DNA methylation of CIMP-associated markers in primary colorectal tumors. BRAF V600E mutations are assumed an early event in serrated pathway of tumourigenesis. 60% of BRAF mutated tumors have association with MSI CRC. BRAF V600E mutations in colorectal carcinoma are connected with older age group, mainly occurs in female gender.[10] It has been revealed that mutation of BRAF such as V600E is closely linked with tumors of proximal colon, mucinous histology and poor differentiation. BRAF mutated tumors are often right sided in contrast to the KRAS mutations which are largely associated with left sided CRC.[11,12] The expression of V600E mutated BRAF can be explored by immunohistochemistry using VE1 i.e. BRAF V600E mutation-specific antibody. Also early screening of BRAF V600E might improve the evaluation of the risks for colorectal cancer and may help in effective management of the patients. IHC additionally offers the benefit of a quicker, faster and easy to perform assay in comparison to molecular testing and it can be successfully and productively used in the diagnostic setting.[13] It has been suggested that immunohistochemical detection of BRAF V600E in routine clinical laboratories can be used as an alternate method to molecular testing and can be recommended as an accurate, easily interpreted and less time consuming technique.

Therefore VE1 immunohistochemistry may act as a helpful tool in the screening for colon carcinomas associated with BRAF mutation but the status of mutation of BRAF should always be validated by molecular genetic studies.[14] Moreover the BRAF V600E mutation has been appraised as an early event in colorectal cancer with multifaceted roles for progression, diagnosis and the prognosis of colorectal cancer.[15]

Limited data is available with regards to the expression of BRAF V600E in colorectal carcinoma in Pakistani population. Hence, this study aimed at evaluating expression of this marker in our population and to correlate it with various clinicopathological features in order to aid selection of effective treatment options.

METHODOLOGY:

This Cross sectional observational study was based on the analysis of colonic biopsies received in the Department of Pathology, PNS Shifa hospital Karachi from March 2016 to March 2019. Ethical approval letter with reference No : ERC 42/2018 was issued by the Ethical Review Committee of Bahria University Medical and Dental College. Informed consent was signed by every patient before enrollment in the study.

The samples were collected including both biopsies and colectomy specimens. Sample size was calculated using software G-POWER (version 3.1.9.2) by taking 95% confidence interval, 5% margin of error. The required sample size was found to be 51. All colonic surgical specimens diagnosed as primary colorectal carcinoma obtained prior to therapy and patients who were willing to participate in the study were included, whereas poorly fixed tissue, inadequate material, metastatic tumors, post radiotherapy specimens as well as patients who refused to participate in the study were excluded from this research.

During the study period, from March 2016 to March 2019, 291 colorectal samples were received at our setup. Both biopsies (n=29) and colectomy specimens (n=22) were analyzed for histopathological diagnosis. Among them 240 cases were reported as benign lesions while 51 cases were diagnosed as colorectal cancer. Hematoxylin and eosin as well as anti-BRAF V600E immunohistochemical staining was performed on the formalin-fixed paraffin-embedded (FFPE) tissues. The clinicopathological data including age, sex, location, microscopic types, and histological grade were collected for statistical analysis. For immunohistochemistry sections of 3 to 5µm thickness were taken from FFPE tumor blocks picked on poly-L-lysine coated slides. was done using retrieval solution (pH 6.0 citrate buffer 10 x) in water bath at 98-99 º C for 40 minutes. Container was removed from water bath and then cooled at room temperature (15 to 20 minutes). Retrieval solution was discarded and section was rinsed two to three times. Endogenous peroxidase was blocked using hydrogen peroxide blocking solution Primary antibody was applied to cover the section. BRAF V600E dilution was done in the ratio of 1:20 as per company provided protocol. After several washing steps in PBS, sections were incubated for 30 min with labeled second antibody. DAB substrate chromogen solution (1 ml substrate buffer + 1 drop DAB chromogen) was applied to cover section, incubated for 2 minutes, washed and counterstained with hematoxylin, dehydrated with ethanol, cleared in xylene and mounted. The slides were then visualized under a light microscope. Tissue samples to which no primary antibody had been added were used as negative controls.

Immunoreactivity was scored by taking into account the percentage of stained tumor cells (Yellow brown color) and intensity of staining. For BRAF V600E, the intensity of cytoplasmic tumor cell staining was scored as weak (1), moderate (2) and strong (3). The cytoplasmic staining of BRAF V600E of at least medium intensity in more than 10% of tumor cells was considered as positive, while the tumors were considered immune negative when there was weak staining or there were less than 10% of stained tumor cells. Papillary thyroid carcinoma with a documented BRAF V600E mutation was used as a positive control. Statistical analysis was done using SPSS version 23.0 Continuous variables were presented as mean and standard deviation. Categorical variables were presented as frequency and percentage. Chi-square and Fisher exact test were used to assess the association of BRAF expression with different clinicopathological parameters. P=0.05 was considered statistically significant.

RESULTS:

In this study 51 cases of colorectal carcinomas were included, among them 16 showed mucinous histology with signet ring cells, 1 showed cribriform pattern, 2 were poorly cohesive tumors, while the rest 32 were adenocarcinomas.

Table-1 showed the immune expression of BRAF V600E protein in cases of colorectal carcinoma. Among the 51 cases subjected to BRAF V600E immunostaining, a total of 20 cases showed positive immune expression for mutated BRAF protein, while remaining 31 cases were negative for BRAF V600E.

Intensity and extent of immune expression of BRAF V600E protein in diagnosed malignant cases of colorectal samples. The positivity was strong (3+) in 7 cases, moderate (2+) in 13 cases-Table-2. The remaining 6 cases showed weak staining intensity with BRAF V600E protein on immunohistochemistry. Total 7 cases revealed strong staining for BRAF V600E protein, 6 cases showing strong reactivity in ?75% of tumor cells and only 1 case showed strong reactivity in almost 50% of tumor cells.

Table-3 correlates the expression of BRAF V600E with different clinicopathological parameters. Out of 37 male patients, 13 cases showed positive expression, while remaining 24 cases showed no expression of BRAF V600E. In female gender 7 out of 14 cases showed no expression of this protein while remaining 7 cases showed positive expression for BRAF V6000. 14 out of 37 left sided lesions showed positive expression of BRAF V600E, while 23 cases were negative for BRAF V600E expression. Among 14 malignant cases from the right colon, 8 cases had no protein expression while remaining 6 cases revealed expression of mutated BRAF protein. 14 out of 32 cases of glandular adenocarcinoma, showed moderate to strong BRAF V600E expression, whereas remaining 18 cases showed no expression.

In this study 16 cases of colorectal carcinoma had mucinous histology with signet ring type cells. Among them 5 cases revealed no protein expression whereas remaining 11 cases showed positive BRAF V600E expression. 2 cases were diagnosed as poorly cohesive and one as having cribriform pattern. Among these only one case of poorly cohesive carcinoma revealed positive BRAF V600E expression on immunohistochemistry.

Table 1: Expression of BRAF-V600E in colorectal carcinoma (n=51)

BRAF-V600E Expression	No of cases of colorectal carcinoma (%)
Positive expression	20 (39.2 %)
Negative expression	31 (60.8%)

Positive expression: cytoplasmic staining of at least medium intensity in more than 10% of tumor cells
Negative expression: tumors were considered immune negative when there was ?10% of stained tumor cells.

DISCUSSION:

This study was aimed to determine the frequency of colorectal cancers received at our setup and to study the expression of BRAF V600E in these cases and to evaluate its effects on colorectal carcinogenesis to select effective treatment options.

In the present study the mean age for colorectal carcinoma was found to be 60.96 years. These findings were in accordance with the figures documented in Shaukat Khanum Memorial Cancer Hospital, Lahore, Pakistan.[16] According to which the estimated mean age for males and females were reported as 53 years and 50 years respectively.[16] A study conducted at Aga Khan University Hospital Karachi in 2014 which included 131 young patients, showed comparatively lower mean age which was documented as 33.3years. This distinction may be attributed to the sample size variation.[17] Similar results were reported in a study showing that colorectal cancer was diagnosed in 65.8% male and 34.2% of female patients.[18]

In the present study most commonly observed grade was well differentiated adenocarcinoma, whereas the common

Table 2: Intensity and extent of BRAF-V600E in diagnosed cases of Colorectal Carcinoma (n= 26)

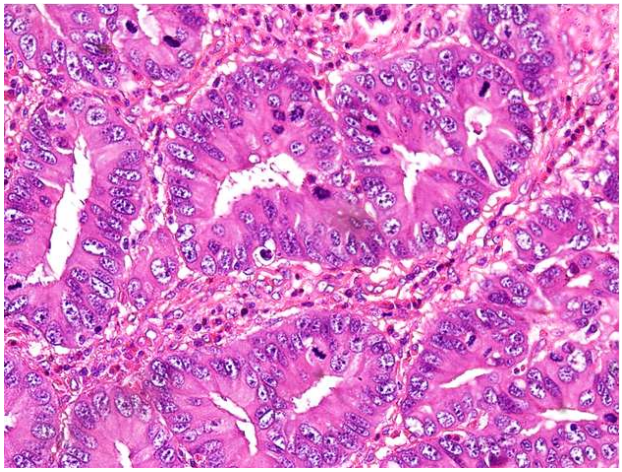
Immunostaining	Extent				Intensity			
	0	1	2	3	0	1	2	3
Weak	1 (16.6%)	2 (33.3%)	1 (16.6%)	2 (33.3%)	0 (0%)	6 (100%)	0 (0%)	0 (0%)
Moderate	0 (0%)	0 (0%)	4 (30.7%)	9 (69.2%)	0 (0%)	0 (0%)	13 (100%)	0 (0%)
Strong	0 (0%)	1 (%)	0 (0%)	6 (87.5%)	0 (0%)	0 (0%)	0 (0%)	7 (100%)

Extent of reactivity (% of immunoreactive nuclei) was as follows: 0, < 10%; 1+, 25-50%; 2+, 50-75%; 3+, >75%.
Intensity of reactivity was as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining

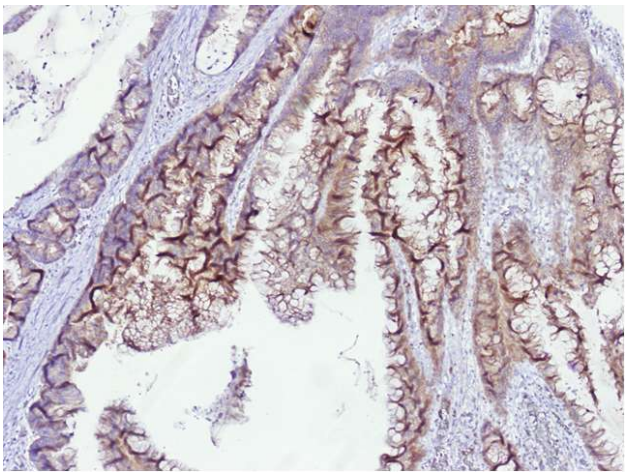
Table 3: Association of clinicopathological features with expression of BRAF-V600E expression (n=51)

Clinicopathogical Features	Total numbers	BRAF V600E		P-value
		Positive	Negative	
Gender	Male = 37	13	24	0.35
	Female = 14	7	7	
Tumor Location	Right-sided =14	6	8	0.758
	Left-sided = 37	14	23	
Glandular Adenocarcinoma	32 (62.7%)	14	18	0.862
Mucinous Carcinoma/Signet ring	16 (31.4%)	5	11	
Poorly cohesive	2 (3.9%)	1	1	
Ciribriform pattern	1 (2.0%)	0	1	

Photomicrograph 1: Colorectal adenocarcinoma H&E X 40



Photomicrograph 2: colorectal adenocarcinoma (same as in photomicrograph 1) showing moderate to strong expression of BRAF V600E in more than 90% of tumor cells. IHC X 20



microscopic variants were reported as adenocarcinoma, and mucinous-signet ring type carcinoma. Our findings corresponded to the figures documented in National Cancer Institute, Cairo University, Egypt (2013) which included 26 metastatic colorectal cancer cases in one study. In this study the histological variation were observed such as, adenocarcinoma, 22(84.6%) cases, mucinous carcinoma, 2(7.7%) cases and signet ring carcinomas, 2(7.7%) cases.[19]

In the present study out of 51 cases of colorectal carcinomas, 37 (72.5%) cases were present in males, while the remaining 14(27.4%) cases of colorectal cancer were seen in females.

With respect to BRAF V600E immune expression, out of 51cases, 20(39.2%) cases showed positive BRAF V600E expression, while remaining 31(60.8%) cases revealed no expression of BRAFV600E on IHC. These results are in agreement with other studies which concluded positive BRAF V600E expression on IHC, as well as on genetic analysis.[14, 20, 21, 22]

In this study we did not find significant correlation of positive expression of BRAF V600E with clinicopathological parameters like, age, gender, location, tumor grades and histological variants. These results are in accordance with a study which did not find any significant correlation between these parameters and BRAF V600E expression.[23, 24]

A study found that tumor stage is important for evaluating BRAF mutant tumors for treatment options. Early tumor stage may be prone to BRAF-specific inhibition alone, as tumor stage advances, various processes must be aimed owing to concentration of mutations. It has been suggested that RAF inhibitor combination strategies can suppress feedback reactivation of MAPK signaling pathway and improve efficacy in BRAF -mutant colorectal cancers.[25]

As the surrounding normal mucosa was also taken into consideration while assessing results of IHC, the study can give an idea regarding the expression of abnormal protein in early lesions also signifying BRAF mutation as a potential early change in tumorigenesis of these cancers.

Last but not the least the presence of BRAF V600E mutation in the current study stresses the need for using anti-BRAF V600E as a routine biomarker by IHC in colorectal carcinoma diagnosis and stresses the significance and importance of BRAF V600E inhibitors as a potential, alternate therapeutic tool in EGFR inhibitor and chemotherapy resistant tumors. The limitations of the study included data from single tertiary

care hospital and small sample size, therefore does not represent the general population. Further large scale multicentric studies will be required to assess the burden of mutations in our population. Additional, relevant clinical data could not be ascertained because of inaccessibility to the record files.ÊIt is strongly recommended that future preferably molecular studies should be conducted to evaluate BRAF V600E mutations as an early carcinogenic event in colorectal cancers. This study also provides a spring board for further studies as it may open venues for exploring new therapeutic options.

CONCLUSION:

BRAF V600E immunoexpression was observed 39. 2% of colorectal carcinoma cases. The expression of BRAF V600E in our population signifies the importance of introducing BRAF V600E as a valuable diagnostic biomarker for colorectal carcinoma. It further stresses the importance of BRAFV600E inhibitors as an alternate therapeutic option in EGFR inhibitor and chemotherapy resistant tumors. Furthermore, the positive BRAF V600E expression in normal mucosa adjacent to the tumor points toward BRAF V600E mutation as an early event in colorectal carcinogenesis.

Author Contribution:
Hina Wasti: Conceived idea, Study designed. Data collection, immunohistochemical analysis, Result interpretation, literature review, Manuscript writing.
Summayyah Shawana: Data analysis, Result interpretation, Proofreading, Manuscript writing & correction of entire Manuscript
Beenish Hussain Nomani: Data collection, immunohistochemical analysis & Proofreading of entire Manuscript
Santosh Kumar Sidhwani: Proofreading of Manuscript & helped to draft the manuscript
Rubbab Mir: Data collection, & helped during immunohistochemical analysis
Hareem Fatima: Proofreading & helped to draft the manuscript

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