RESEARCH ARTICLE

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KRAS and *BRAF* Hotspot Variants Show Lower Prevalence in Bangladeshi Colorectal Cancer Patients

Muhammad Sibgatullah Zunnun[1,2], Md. Zahin Alam[1,2], S. M. Mahbubur Rashid[1], Md. Shihab Al Mashiur Rahaman[3], Nahid Parvez[1], Md. Shahadot Hossain Sheikh[3], Farida Arjuman[4], Mustak Ibn Ayub[1]*

Abstract

Objective: Colorectal cancer (CRC) is the second-leading cause of cancer-related mortality globally, with a rising incidence in Bangladesh. This study aims to identify pathogenic *KRAS* and *BRAF* mutations contributing to anti-EGFR resistance in CRC patients. **Methods:** Two sets of primers were used: one was adopted from a published paper, and another was designed using Primer3Plus. They were optimized for specific PCR amplicons of 173 bp and 230 bp from exon 2 of *KRAS* and exon 15 of *BRAF*, respectively. Targeted Sanger sequencing was performed to analyze mutations in these amplicons in 46 CRC patient samples. **Results:** *KRAS* mutations were detected in 5 samples (10.87%), comprising two G12D, one G12V, and two G13D variants. Notably, no *BRAF* V600E mutation was identified. These results stand in sharp contrast to global data, where *KRAS* mutations at these loci occur in approximately 40% of cases and *BRAF* V600E mutations in about 10%. Such mutations are clinically significant because they confer resistance to anti-EGFR therapy, underscoring the distinct molecular profile observed in this cohort. **Conclusion:** The lower prevalence of *KRAS* and *BRAF* mutations indicates the potential effectiveness of anti-EGFR therapies in Bangladeshi CRC patients than other populations.

Keywords: Colorectal cancer, *KRAS, BRAF*, Anti-EGFR resistance, Targeted Sanger sequencing

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Introduction

Colorectal cancer (CRC) poses a worldwide challenge because of its high occurrence and mortality rate. According to the GLOBOCAN 2022 data, colorectal cancer is the third most commonly diagnosed cancer, accounting for 1.9 million new cases (9.6%), and ranks second in terms of cancer-related mortality with 904,000 deaths (9.3%) globally, with the rates projected to be increased in the upcoming years [1]. As CRC is a heterogeneous disease with a complex molecular profile, understanding the molecular mechanisms and epidemiology of this disease is crucial in its diagnosis and treatment.

Pathogenic mutations play a pivotal role in the initiation and progression of various cancers, contributing to the dysregulation of critical cellular pathways. *KRAS* and *BRAF*, both members of the *RAF-MEK-ERK* signaling pathway, are frequently mutated in diverse cancer types. These mutations result in aberrant activation of downstream signaling cascades, promoting uncontrolled cell proliferation, survival, and metastasis [2, 3].

KRAS mutations are among the most common genetic alterations in human cancers, occurring in various malignancies, including colorectal, pancreatic, and lung cancers [4, 5]. The most important mutations in exon 2 are *G12D* (Glycine to Aspartic Acid at codon 12), *G12V* (Glycine to Valine at codon 12), *G13D* (Glycine to Aspartic Acid at codon 13), and G12C (Glycine to Cysteine at codon 12). *KRAS* normally functions as a molecular switch that cycles between an inactive GDP-bound state and an active GTP-bound state, tightly regulating downstream signaling. However, codon 12/13 mutations impair the intrinsic GTPase activity of *KRAS*, preventing the conversion of GTP to GDP and locking the protein in a constitutively active GTP-bound form. These mutations, present in approximately 40% of CRC patients globally, lead to continuous activation of downstream effectors, such as the mitogen-activated protein kinase (MAPK) pathway, thereby promoting oncogenic transformation and resistance to programmed cell death [6, 7]. *KRAS* mutations are associated with a poorer prognosis in certain cancers, including colorectal cancer and non-small cell

*[1]Department of Genetic Engineering and Biotechnology, University of Dhaka, Dhaka, Bangladesh. [2]Department of Biotechnology and Genetic Engineering, United International University, Dhaka, Bangladesh. [3]Bangladesh Medical University (BMU), Dhaka, Bangladesh. [4]National Institute of Cancer Research and Hospital (NICRH), Dhaka, Bangladesh. *For Correspondence: miayub@du.ac.bd*

lung cancer (NSCLC) [8]. In colorectal cancer, *KRAS* mutations are a crucial predictive marker for response to anti-EGFR (epidermal growth factor receptor) therapies, such as cetuximab and panitumumab, as patients with *KRAS* mutations are generally resistant to these therapies. *KRAS* mutation diagnosis influences treatment decisions and helps oncologists avoid ineffective anti-EGFR therapies in patients with mutated *KRAS* [9].

BRAF, a serine/threonine kinase that operates downstream of *KRAS*, is another key player in the *RAF-MEK-ERK* pathway. The V600E mutation (a valine-to-glutamic acid substitution at codon 600), a hallmark *BRAF* mutation, is present in 10% of all CRC patients. This mutation results in the hyperactivation of *BRAF* and subsequent dysregulation of downstream signaling. *BRAF* mutations are prevalent in various cancers, such as colorectal cancer and melanoma [10, 11]. *BRAF* mutations are also associated with a poorer prognosis in colorectal cancer and more aggressive disease courses. Similar to *KRAS*, patients with *BRAF* mutations in colorectal cancer are less likely to benefit from anti-EGFR treatment. In melanoma and some other cancers, *BRAF* mutations are directly targeted with *BRAF* inhibitors, such as vemurafenib and da*BRAF*enib [12]. However, the use of *BRAF* inhibitors in colorectal cancer is more complex, and their efficacy may be influenced by other genetic factors [13]. So, identifying *KRAS* and *BRAF* mutations plays a crucial role in cancer treatment, especially with the rise of precision therapies.

Identifying these *KRAS* and *BRAF* mutations facilitates early cancer detection, predicts prognosis, and therapy responses. These are parts of the broader molecular diagnostics tests that help in developing personalized treatment strategies, ensuring that therapies specifically address the unique characteristics of each patient's cancer by identifying driver mutations that support the survival of cancer cells. These therapies function by targeting the specific proteins or pathways involved, enhancing the precision and efficacy of the treatment [14]. By having knowledge of the unique mutations profile of a cancer, it helps the clinicians to select therapies that are more likely to be effective by predicting therapy responses and their optimal dosage, while also reducing adverse effects. When compared to conventional cancer treatments that are universally applicable and do not account for inter-individual variability, this represents a significant advancement [15].

In Bangladesh, CRC incidence is increasing, but molecular profiling data remain limited and mostly hospital-based. The few available Bangladeshi studies report variable but generally lower frequencies of canonical anti-EGFR resistance markers than global estimates; for example, a Bangladeshi cohort (n=44) detected *KRAS* mutations in 31.5% and *BRAF* in 4.85% of tumors [16], while another recent Bangladeshi cohort (n=30) reported *KRAS* mutations in 16.7% and no *BRAF* mutations [17]. Similar trends have been reported in Bangladeshi-origin populations abroad, where a study on British Bangladeshi CRC patients (n=29) identified a low *KRAS* mutation frequency (5.4%) and absence of *BRAF* mutations compared to higher rates in Caucasian populations [18]. These findings highlight the need for additional, population-specific datasets to define the actionable mutation landscape in Bangladeshi CRC patients. In this study, we aim to determine and validate the incidence of two well-established predictive markers of colorectal cancer, which are mutations in the *KRAS* and *BRAF* genes, through the molecular biology technique known as targeted Sanger sequencing. This research holds translational value, serving as a reference for determining the most effective treatment plans for individual patients in Bangladesh.

Materials and Methods

Collection of colorectal tissue samples

A total of 46 tissue samples from CRC patients were collected from the Department of Colorectal Surgery, Bangladesh Medical University (BMU), and National Institute of Cancer Research and Hospital (NICRH), Bangladesh. In the presence of an experienced pathologist, cancerous tissue was identified by its solid, mass-like growth pattern and the absence of the well-defined crypt structures characteristic of normal colon and rectal tissue.

Genomic DNA Extraction

DNA was extracted from the fresh tissue and/or tissue preserved in -20 ºC, using QIAamp® DNA Mini Kit (QIAGEN, Germany) following the manufacturer's protocol. Then, DNA concentration and quality were measured using a NanoDrop® spectrophotometer, and the extracted DNA was visualized in 1% agarose gel electrophoresis. The accepted genomic DNA purity range was between 1.8-2.0.

Polymerase Chain Reaction

A previously published primer set was used to amplify *KRAS* exon 2 (codon 12/13) [19], while a primer set was designed for *BRAF* exon 15 (codon 600), both of which are frequently mutated in colorectal cancer with anti-EGFR resistance. Target gene sequences were retrieved from the UCSC Genome Browser (GRCh38), and primers were designed using Primer3Plus [20], following criteria such as 18–26 bp length, 40–60% GC content, and avoidance of homopolymeric stretches. Primers were commercially synthesized, dissolved in nuclease-free water (HyPureTM) to 100 µM stock, and diluted to 10 µM working concentration before storage at -20°C. UCSC in silico PCR validated primer specificity, and gradient PCR determined optimal annealing temperatures. Primer sequences and annealing temperatures are listed in Table 1.

PCR reactions were set up using 12.5 µL GoTaq® Green Master Mix, 0.5 µL of each primer, 100 ng of template DNA, and required nuclease-free water to a final volume of 25 µL. Amplifications were performed on a ProFlex PCR system under the cycling conditions: initial denaturation at 95 °C for 4 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at variable temperature for each gene for 30 s, and extension at 72 °C for 45 s; followed by final extension at 72 °C for 5 min. Amplicons were resolved on 1% agarose gel electrophoresis for specific bands.

Table 1. Oligonucleotide Primers Used for *KRAS* and *BRAF* Mutation Analysis

Gene	Primer Sequence	Tm (°C)	Product Size(bp)
KRAS	F-AAGGCCTGCTGAAAATGACTG	60	173
(E2)	R- CAAAGAATGGTCCTGCACCAG		
BRAF	F- TGCTTGCTCTGATAGGAAAATGAGA	62	230
(E15)	R- GCAGCATCTCAGGGCCAAAA		

F, Forward; R, Reverse; E, Exon)

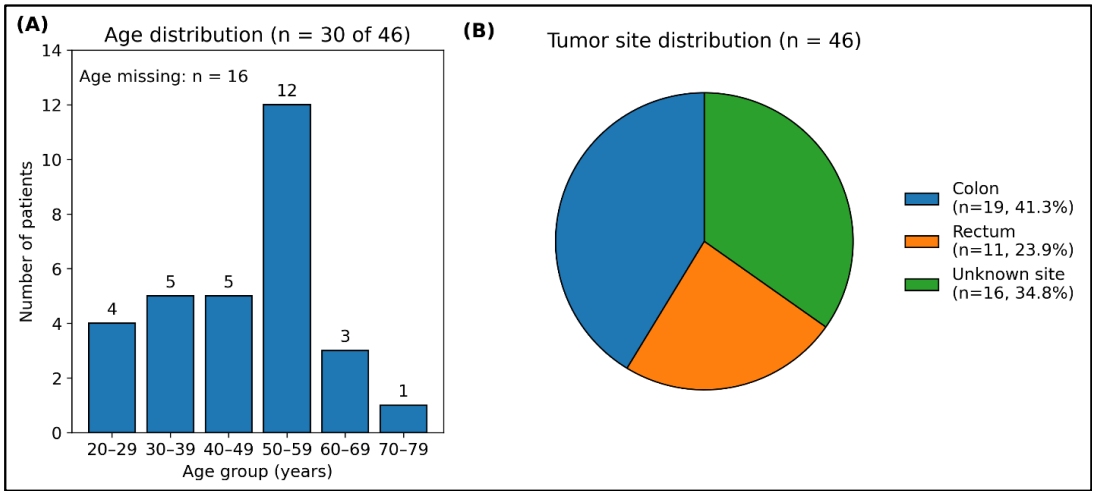

Figure 1. Age Distribution and Tumor Site of Colorectal Cancer Patients. (A) Age distribution of patients with known age (n = 30), showing the highest frequency in the 50–59 age group (n = 12). Fourteen patients were younger than 50 years. (B) Tumor site distribution among all patients (n = 46): colon (41.3%, n = 19), rectum (23.9%, n = 11), and unknown site (34.8%, n = 16).

Targeted Sanger Sequencing

Targeted Sanger sequencing of the *BRAF* and *KRAS* PCR amplicons was outsourced from the ICDDR,B Genome Center, Bangladesh, Child Health Research Foundation (CHRF), Bangladesh, and Center for Advanced Research and Sciences, University of Dhaka, Bangladesh. To analyze sequencing data, Chromas was used for visualizing nucleotide variations in *KRAS* and *BRAF*. This allows for the confirmation of somatic mutations by examining chromatogram peaks.

Statistical analysis

The statistical and mathematical analysis as well as graphical representations were performed using MS Excel and the programming language Python.

Results

Demographic and clinicopathological data of CRC patients

This study included 46 colorectal cancer patients; their demographics are summarized in Table 2. Among them, 14 were male, 16 were female, and the gender status of 16 patients was unknown.

All patients were clinically diagnosed with either primary or metastatic colorectal cancer. They received adjuvant chemotherapy followed by surgical intervention. The patients' ages ranged from 25 to 72 years, with a peak in the 50–59 age range (n=12). The mean age was 46.67±12.21. Fourteen patients were below 50 in age, indicating early-onset colorectal cancer. Nineteen patients had colon cancer, while eleven had rectal cancer (Figure 1). The exact tumor locations in the colon were the ascending colon, sigmoid colon, descending colon, caecum, hepatic flexure, and splenic flexure for the known cases.

PCR amplification of the hotspot regions of KRAS and BRAF

KRAS and *BRAF* gene regions linked to colorectal cancer were amplified by PCR using the primer pairs listed in Table 1. Each of the amplicons showed specific bands

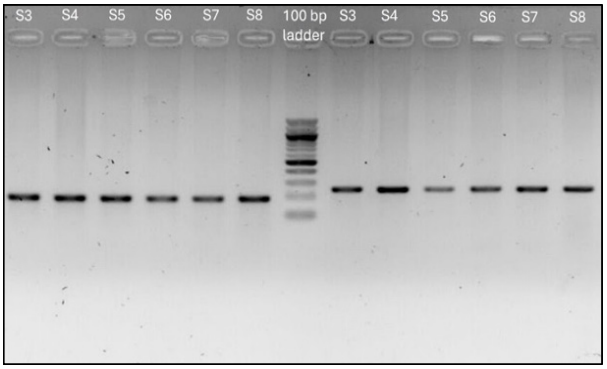

Figure 2. Visualization of *KRAS* and *BRAF*-specific PCR amplicons in agarose gel. Specific *KRAS* (173 bp) and *BRAF* (230 bp) bands were observed on the left and right side respectively of the 100 bp DNA ladder (New England Biolabs). Samples S3–S8 represent colorectal cancer (CRC) cases.

Table 2. Clinicopathological Characteristics of the Study Cohort (n = 46)

Characteristics	Attribute		Count	Percentage
Age	<50		14	30.44
	50-64		14	30.44
	>65		2	4.34
	Unknown		16	34.78
Gender	Male		14	30.44
	Female		16	34.78
	Unknown		16	34.78
Tumor Location	Rectum		11	23.91
	Colon	Hepatic Flexure	5	41.31
		Splenic Flexure	5	
		Ascending Colon	4	
		Sigmoid Colon	2	
		Descending Colon	2	
		Caecum	1	
	Unknown		16	34.78
Metastasis	Yes		5	10.87
	No		25	54.35
	Unknown		16	34.78

in agarose gel with the desired product size (Figure 2).

KRAS and BRAF mutations detection by targeted Sanger sequencing

Targeted sequencing of the 46 PCR amplicons was performed from our collaborative and commercial partners. The sequence quality was consistent and discernible for all 46 samples when analyzed using Chromas software. In the chromatogram, a double peak was expected in the mutated region. In each mutation-containing sample, the heights of the mutant and wild-type peaks differed, reflecting the proportion of mutation-carrying cells consistent with the

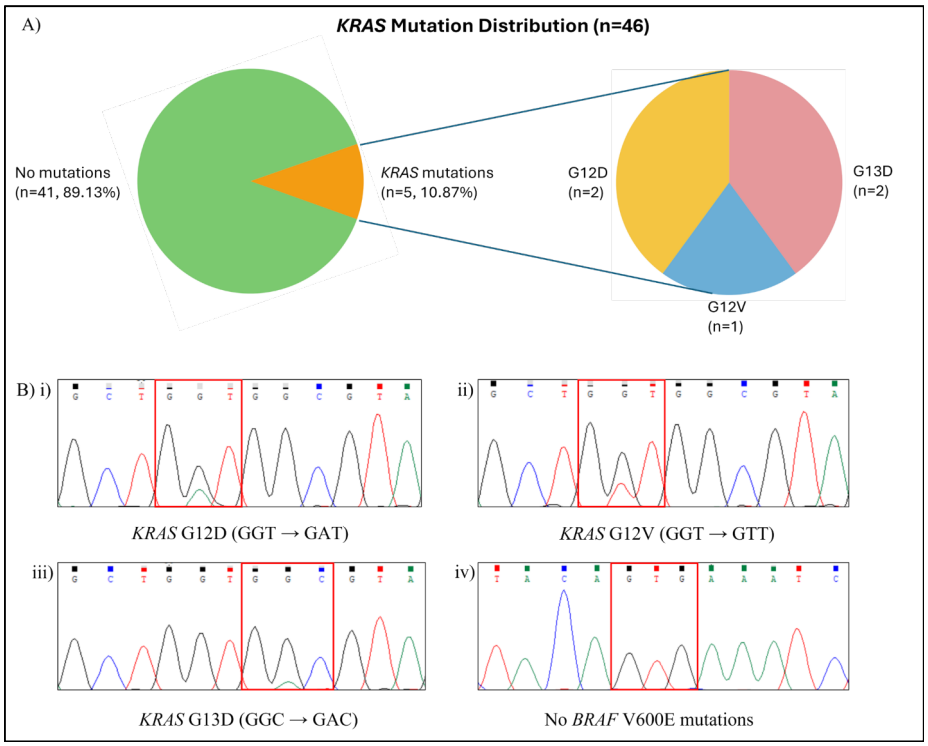

Figure 3. Targeted Sanger Sequencing Results for the Detection of Somatic *KRAS* and *BRAF* mutations. (A) Distribution of *KRAS* and *BRAF* mutations among the 46 samples, showing 10.87% positive for *KRAS* somatic mutations (n=5) and 0% for *BRAF* mutations. The specific *KRAS* mutations identified were G12D (n=2), G12V (n=1), and G13D (n=2). (B) Representative chromatograms demonstrating somatic mutations: (i) *KRAS* G12D (GGT → GAT), (ii) *KRAS* G12V (GGT → GTT), (iii) *KRAS* G13D (GGC → GAC), and (iv) a *BRAF* sample negative for V600E mutations. The presence and relative abundance of somatic mutations are indicated by double peaks of varying heights.

somatic nature of the mutations (Figure 3).

A careful analysis showed that only 5 (10.87 %) samples had *KRAS* somatic mutations, while no *BRAF* mutations were observed. Among these five *KRAS* mutations, two were *G12D*, one was *G12V*, and two were *G13D* mutations.

Discussion

CRC is one of the leading causes of death globally. Molecular diagnostics allows the identification of key genetic alterations that drive CRC progression and treatment responses. Among these, *KRAS* and *BRAF* mutations are identified globally in approximately 40% and 10% of CRC cases, respectively. They are linked to the abnormal signaling pathway leading to the development of tumors. Mutations in both of the genes are associated with anti-EGFR therapy resistance, thus influencing the treatment decisions [9, 10]. Therefore, in the field of precision oncology, the significance of these mutations is enormous.

In this study, 46 samples have been analyzed so far. Among them, 5 patients had somatically mutated *KRAS* exon 2 in codons 12 and 13. No *BRAF* mutations were found in the 230 bp amplicon. In our research, the percentage of *KRAS* mutations in our colorectal cancer patients was 10.87%, which is actually lower than the global scenario (40%) [21]. These findings on *KRAS* and *BRAF* mutations align with the study done in London in the year 2013 at Queen Mary University. They compared mutation patterns in Caucasian patients and British Bangladeshi patients. They found 5.4% *KRAS* mutations and no *BRAF* mutations in 29 British Bangladeshi MSS CRC patients as compared to 25% and 10% for the respective gene mutations in 133 Caucasian MSS CRC patients [18].

This study shows that the prevalence of *KRAS* and *BRAF* mutations is low in the Bangladeshi population. These mutations serve as critical predictive markers, guiding oncologists away from anti-EGFR treatments that would likely be ineffective. But if the patients do not carry this mutation, they will benefit from chemotherapy treatment with Cetuximab, Panitumumab. Thus, our study indicates a better efficacy of anti-EGFR therapy in Bangladeshi patients. Moreover, the low prevalence of these mutations in the Bangladeshi population suggests that there may be other mutations that drive the development of colorectal cancer in this population, which should be studied further.

However, several limitations should be considered when interpreting the findings of this study. The relatively small sample size and single-center sample collection may limit statistical power and reduce the generalizability of the results to the broader Bangladeshi population. The cross-sectional nature of the study, without longitudinal clinical follow-up, restricts the ability to assess the prognostic or therapeutic impact of the detected mutations. In addition, the analysis was confined to hotspot regions of *KRAS* and *BRAF*, which may have resulted in missed mutations outside these regions. Future studies incorporating larger, multi-center cohorts, broader genomic approaches such as next-generation sequencing, and longitudinal clinical data will be essential to validate and extend these observations, ultimately contributing to more effective precision oncology strategies in Bangladesh.

Author Contribution Statement

M.I.A. – Conceptualization; M.I.A. and S.M.M.R. – supervision and planning; M.S.Z., M.Z.A. and N.P. – experiment conduction and formal analysis; M.S.Z. – manuscript writing and original draft preparation; M.I.A. and S.M.M.R. – manuscript review and finalization; M.S.A.M.R., M.S.H.S. and F.A. - Clinical data collection and sample selection. All authors have read and agreed to the published version of the manuscript.

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Ethical approval

The ethical approval for this research was obtained from the Ethical Review Committee of the University of Dhaka (Ref. No.257lBiol. Scs.).

Conflict of interest

The authors declare no competing interests.

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