

Gastrointestinal Cancer

The Prevalence of BRAF, PIK3CA, and RAS Mutations in Indian Patients with Colorectal Cancer

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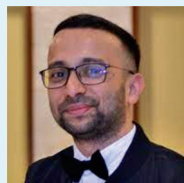
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Abstract



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Keywords

- ▶ colorectal cancer (CRC)
- ▶ India
- ▶ BRAF
- ▶ PIK3CA
- ▶ RAS

Introduction The present study evaluates the mutation pattern and frequency of *BRAF*, *PIK3CA* and *RAS* in colorectal carcinoma observed in the tertiary cancer center in India.

Materials and Methods Consecutive cases of colorectal adenocarcinoma ($n = 330$) registered from January 2015 to December 2019 (5-year duration) were selected for the study. Molecular analysis for *BRAF*.*PIK3CA* (exon 9 and 20) and *RAS* (*KRAS*&*NRAS*) was performed on representative formalin-fixed paraffin-embedded tissues by Sanger sequencing. Results were correlated with clinicopathological features. Patient overall survival (OS) was obtained using Kaplan–Meier method.

Results The study cohort was in the age range of 22 to 81 years (median age: 52 years) that included 202 males and 96 females (male: female ratio 2.1:1). *BRAF* V600E mutation was observed in three cases (1%), while 17 cases (5.7%) had mutations in the *PIK3CA* gene (exon 9 or exon 20). Mutation analysis for *RAS* gene (*KRAS*&*NRAS*) was observed among 42 (15.4%) cases with *KRAS* mutation and 11 (4%) cases were positive for *NRAS* mutations. Among *RAS*, *KRAS* G12D was the predominant mutation. Median OS with wild-type *RAS* was 46.6 months (95% confidence interval [CI]: 22.4–70.8), while for *RAS* mutated patients, it was 25.6 months (95% CI: 16.7–34.5), hazard ratio: 1.7 (95% CI: 1.1–2.7, $p = 0.025$).

Conclusion This study evaluated the prevalence of *BRAF*, *PIK3CA* and *RAS* mutations in the Indian cohort and its impact on clinical behavior. There was lower incidence of *BRAF* mutations in this cohort and *PIK3CA* mutation (single) did not impact survival of the patients.

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Introduction

The present study evaluates the mutation pattern and frequency of *BRAF*, *PIK3CA*, and *RAS* in colorectal cancer (CRC) observed from a tertiary cancer center in India.

Materials and Methods

Sample

This study was approved by the Institutional Ethics Committee (vide letter number OIEC/1015/1578/0001) of the Tata Memorial Hospital, Mumbai.

Consecutive cases of colorectal adenocarcinoma ($n = 330$) registered at the Gastrointestinal Department, Tata Memorial Hospital from January 2015 to December 2019 (5-year duration) were screened for the availability of representative tumor specimen, clinical data, and tumor adequacy ($\geq 20\%$). From these 330 cases, 298 have been included in this study.

Molecular analysis was performed on representative formalin-fixed paraffin-embedded (FFPE) tissues. Demographic and clinicopathological details for each case were collected from the Electronic Medical Record of the Hospital. Waiver of informed consent was obtained as this is a retrospective observational study.

Immunohistochemistry

Immunohistochemistry was performed for microsatellite instability (MSI; PMS2, MLH1, MSH6, MSH2), CK20, CDX2, and CK7 on an automated immunostainer (Benchmark XT, Ventana Medical Systems Inc., Tucson, Arizona, United States). Details of the various antibodies, including company name and dilution, are enlisted in [–Supplementary Table S1](#) (available online only).

DNA Extraction

Tissue sections ($5 \times 10 \mu\text{m}$) obtained from FFPE blocks were deparaffinized with limonene (Sigma Aldrich, United States, USB, Affymetrix, ThermoFisher scientific, USA) followed by overnight digestion and DNA extraction with QIAamp DNA mini kit (Qiagen, United States) as per manufacturer's instructions. The extracted DNA was then checked for quality (260:280 ratio) and quantity using ultraviolet spectrophotometer (Thermo Scientific NanoDrop 2000). The integrity of the extracted DNA was assessed by *ACTNB* gene (housekeeping gene) polymerase chain reaction (PCR) (sequence mentioned in [–Supplementary Table S2](#), available online only). Only those samples that showed amplification for the *ACTNB* gene were selected for *RAS*, *BRAF*, and *PIK3CA* PCR.

PCR Amplification and Cycle Sequencing

BRAF, *PIK3CA* (exon 9 and 20) and *RAS* (*KRAS*, *NRAS*) genes were amplified each using 100 ng of template DNA with respective primers. Primer details, product size, and the PCR conditions for each of the above-mentioned genes are mentioned in [–Supplementary Table S2](#) (available online only). Amplified products were enzymatically purified using EXO-SAP-IT (USB, Affymetrix) and subjected to cycle sequencing. Sequencing was performed on the purified PCR products

with BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Thermo scientific, United States) followed by purification using Optima DTR (Edge BioSystems, United States). The purified products were sequenced on ABI3500 Genetic Analyzer (Applied Biosystems, United States).

Sequence Analysis

Sequencing results were analyzed using sequence analysis software Chromas Lite Version 2.6.6. The mutations were reported as per the recommendations of the Human Genome Variation Society (HGVS).

As the limit of detection of Sanger sequencing was 5%, to ensure no false-negative data was obtained, real-time-PCR (RT-PCR) assay was employed to detect the *BRAF* V600E alterations.

Real-Time PCR (RT-PCR for BRAF)

Real-time PCR assay was used to detect *BRAF* V600E mutations using TaqMan Gene Expression master mix on AB Quant Studio 12 Flex System (Thermo Scientific, United States). The sequence for wild-type *BRAF* and *BRAF* V600E probe was designed as per literature (3). The assay was set up in triplicates for both the genotypes as per cycling conditions mentioned in [–Supplementary Table S3](#) (available online only). Results were analyzed on expression suite software from Quant studio (Thermo Scientific, United States).

Statistical Methods

Molecular results were correlated with clinicopathological features, namely age, sex, primary tumor site and laterality, MSI status using IBM SPSS Statistical software 25.0. The data were summarized using descriptive statistics. Data pertaining to continuous variables, such as age, were described using the mean \pm standard deviation of the median (range) for normally distributed data. Patient overall survival (OS) was calculated from the date of registration till the date of last follow-up using the Kaplan–Meier method.

Results

Among the 330 cases selected for the study, molecular and clinical data could be obtained only for 298 cases.

Clinicopathological Features

As Tata Memorial Hospital is the tertiary cancer center, the cases included people from across the country. The median age of the study cohort was 52 years (range: 22–81 years). The overall male: female ratio was 2.1:1.

Primary site of disease was known for 295 cases. In 238 cases (80.7%), the primary site of disease was observed in left colon, while the remaining 57 cases (19.3%) presented with right colon as the primary site. Nearly 55% of the cases were stage IV disease ([–Supplementary Fig. S1](#), available online only).

Immunohistochemistry

CK20 marker was positive in 83% of the cases ($n = 35/42$), while 79% cases were positive for CDX2 ($n = 34/43$). Details regarding MSI status were available for 107 cases, with 95 cases being MMR proficient (88.8%), while 12 cases (11.2%)

were MMR deficient. MSI status was correlated with clinicopathological features and molecular alterations (**–Supplementary Table S4**, available online only). However, no statistical significance was observed. Among the three cases showing *BRAF* V600E mutation, two cases were MMR proficient, while MSI status was not known in one case.

Molecular Analysis

Consecutive cases of CRC ($n=298$) were included in this study to analyze gene alterations (**–Supplementary Fig. S2**, available online only) in *BRAF*, *PIK3CA* (exon 9 and 20) and *RAS* (*KRAS*, *NRAS*, *HRAS*).

BRAF Gene Alteration

BRAF gene mutation analysis could be performed in 298 cases, of which 20 cases (6.7%) were uninterpretable due to noisy sequencing data. *BRAF* V600E mutation was observed in three cases (1%), all of which were metastatic. There was 100% concordance observed between Sanger sequencing and real-time PCR to detect *BRAF* V600 alterations.

PIK3CA Gene Alterations

Among the 298 cases screened for *PIK3CA* gene alterations, 17 patients (5.7%) had mutations in the *PIK3CA* gene (exon 9 or exon 20); out of these, 10 (58.9%) had metastatic disease.

PIK3CA Exon 9

Mutations in *PIK3CA* exon 9 gene were observed in 14 cases (4.7%) that included single-nucleotide variants (SNV) ($n=13$) and frame-shift mutation ($n=1$). Among the cases harboring a mutation in the *PIK3CA* exon 9 region, nine (64.3%) cases were females, while the remaining five cases (35.7%) were males.

The frame-shift mutation seen and 11 SNVs (E545Q [$n=5$], E545K [$n=1$], Q546P [$n=3$], p.P539= $[n=2]$) were associated with stage IV disease, while SNVs E545G ($n=1$) and E542Q ($n=1$) were associated with stage IV disease.

PIK3CA Exon 20

Mutations in *PIK3CA* exon 20 were observed in three cases (1%). All were substitution mutation R1023* ($n=1$), R1023Q ($n=1$), R1034* ($n=1$) associated with stage IV disease. Synonymous p.T1025= (c.3075C>T) SNV were observed in 27 cases. No case of coexistent exon 9 and 20 mutation was found in this study.

RAS Gene Alterations

Mutation analysis for the *RAS* gene (*KRAS* and *NRAS*) could be obtained in 273 cases only; the remaining cases were uninterpretable due to sequencing artifacts. The correlation of *RAS* gene status with clinicopathological features is given in **–Supplementary Tables S5** and **S6** (available online only).

KRAS Gene

Among these 273 cases, 42 (15.4%) cases were positive for *KRAS* mutation. Usual mutations were in the *KRAS* exon 12, namely p.G12D ($n=15$, 35.7%), p.G12V ($n=6$, 14.3%), p.G12C ($n=5$, 11.9%), and p.G12S ($n=4$, 9.5%). Other mutations observed in *KRAS* gene were p.G13D ($n=3$, 7.14%), p.Q61L ($n=2$, 4.76%), p.G12A

($n=2$, 4.76%), p.A146T ($n=1$, 2.38%), p.Q61H ($n=1$, 2.38%), p.A131T ($n=1$, 2.38%), p.G13S ($n=1$, 2.38%), p.G13C ($n=1$, 2.38%), and p.A134V ($n=1$, 2.38%) (**–Supplementary Fig. S3**, available online only) One patient had complex mutation of *KRAS* exon 2: c.35G>A (p.G12D), and *HRAS* exon 3: c.181C>T (p.Q61*).

Twenty-eight patients (25.2%) out of 111 stage IV CRC patients undergoing a successful *KRAS* testing had a mutation in this gene. Patients with mutations in the *KRAS* gene were exclusive for mutations in *BRAF* or *PIK3CA* genes.

NRAS Gene

NRAS gene alterations were observed in 11 cases (9.92%), predominantly in exon 2 viz p.G12V ($n=5$; 45.4%). Other mutations observed in exon 2 included p.G13A ($n=1$; 9.1%), p.G13V ($n=1$; 9.1%), and p.A149T ($n=1$; 9.1%). Mutations observed in exon 3 included p.A59V ($n=1$; 9.1%), p.Q61K ($n=1$; 9.1%), and p.Q61L ($n=1$; 9.1%). Thus, the total incidence of *RAS* mutations in stage IV disease was 35.1%. Mutations observed in *BRAF* and *RAS* were mutually exclusive.

Correlation of Tumor Laterality with Gene Alterations

Among the 298 cases included in the study, 238 patients (80.7%) had left-sided CRC, while 57 patients (19.3%) had right-sided CRC (**–Supplementary Table S7**, available online only). The primary site was not known for 3 (1%) cases. MSI status was correlated with tumor laterality. MSI instability was observed in eight patients with the primary site in the left colon and four patients with the primary site in the right colon.

BRAF gene mutations were predominantly observed in the right colon ($n=2/3$; 66.6%). *PIK3CA* exon 9 mutation was observed in 14 cases, of which 11 (78.6%) cases had left-sided CRC, while three cases (21.4%) had right-sided CRC; whereas all three patients harboring *PIK3CA* exon 20 mutations had left-sided CRC. Among the patients harboring *KRAS* gene mutation, 30 cases (71.4%) had left-sided colorectal cancer, while 11 cases (26.2%) were right-sided CRC. The primary site was not known for one case.

Overall Survival

Out of 298 patients, survival data was available for 277 patients. The median follow-up was 30.8 months (range: 0–355) for the entire cohort.

Median OS with wild-type *RAS* was 46.6 months (95% confidence interval [CI]: 22.4–70.8), while for *RAS* mutated patients, it was 25.6 months (95% CI: 16.7–34.5), hazard ratio: 1.7 (95% CI: 1.1–2.7, $p=0.025$) (**–Table 1**).

Survival data could be obtained among 241 patients with *PIK3CA* analysis and among 262 patients with *BRAF* analysis. Median OS with wild-type *PIK3CA* was 40.8 months (95% CI: 29.0–52.7), while for *PIK3CA* mutated patients, it was not reached ($p=0.310$). Median OS with wild-type *BRAF* was 44.4 months (95% CI: 25.1–59.3), while for *BRAF* mutated patients, it was 19.2 months (95% CI: 3.5–34.8) ($p=0.488$).

Discussion

The present study evaluates the molecular alterations in *BRAF*, *PIK3CA*, and *RAS* (*KRAS* and *NRAS*) genes in 330

Table 1 Overall survival of the patients according to the mutations and its correlation with stage and sidedness

OS in months with 95% CI	RAS mutated	RAS wild type	PI3K mutated	BRAF mutated
Stage IV	(n = 30) 25.6 months (95% CI: 11.4–39.7)	(n = 79) 46.6 months (95% CI: 22.4–70.8)	(n = 12) 40.8 months (95% CI: 16.2–65.4)	(n = 3) 19.1 months (95% CI: 3.4–34.8)
Right sided	(n = 10) 15.3 months (95% CI: 8.9–21.8)	(n = 34) NR	(n = 3) NR	(n = 2) 19.1 months (95% CI: 3.4–34.8)
Left-sided	(n = 24) 25.6 months (95% CI: 15.0–36.1) $p = 0.460$	(n = 45) 14.1 months (95% CI: 0–48.7) $p = 0.752$	(n = 13) NR	(n = 1)

Abbreviations: CI, confidence interval; NR, not reached; OS, overall survival.

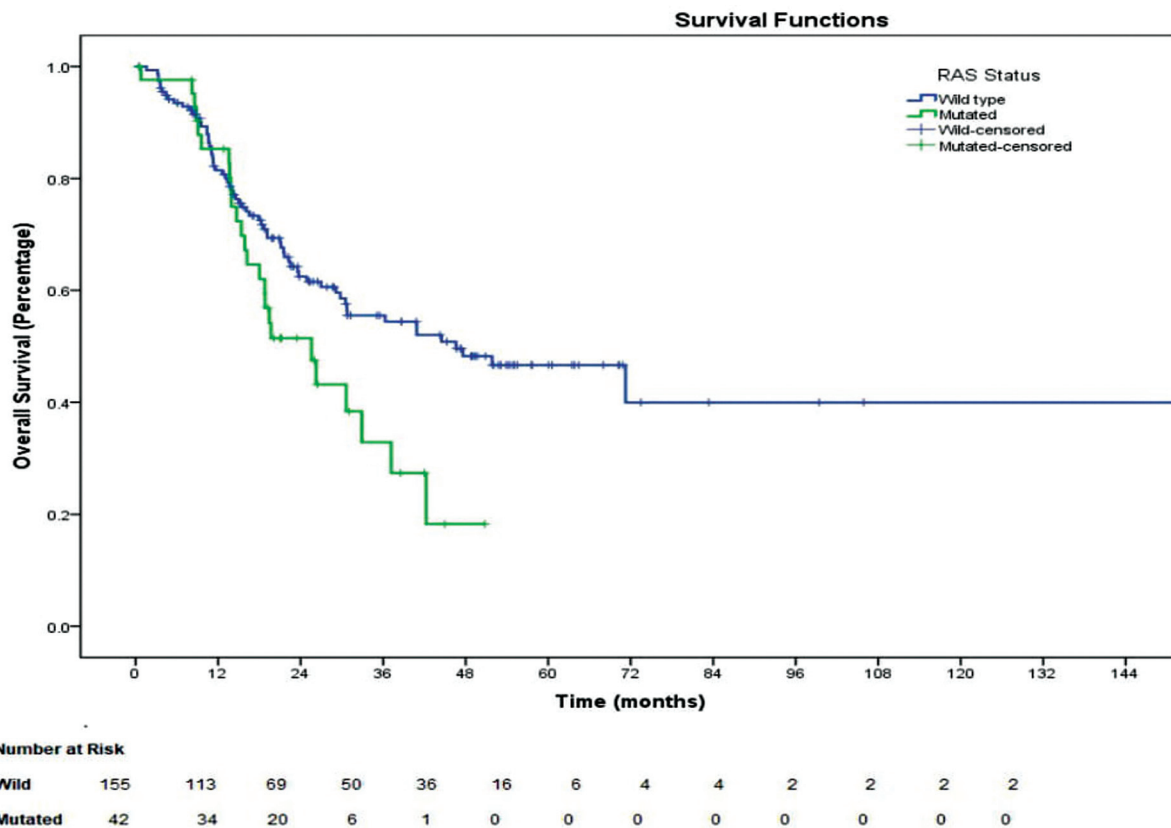


Fig. 1 Overall survival of RAS wild versus RAS mutant cohort.

consecutive cases of CRC cases reported from a tertiary cancer center in India. Along with the molecular profiling, the present study also incorporates the survival data. RAS gene alterations were more frequent (19.4%) followed by PIK3CA (5.7%) and BRAF (1%). BRAF and RAS mutations were mutually exclusive. Previous studies on the prevalence of BRAF mutations in CRC from the West have revealed a mutation frequency of 5 to 20%,^{1,2} while studies from the Asian continent have reported a slightly lower frequency of 4 to 14%.³⁻⁶ A very low frequency of BRAF alteration was observed in this cohort that could indicate alternate key driver mutations responsible for disease progression. The median OS in patients harboring BRAF V600E was found to be ~19.2 months ($p = 0.488$) in this series.

KRAS mutation frequency observed in the current cohort was lower than that reported in the literature. Previous studies have reported KRAS and NRAS mutation frequency of ~35 to 50% and 6 to 10%, respectively.⁷ In our study, the OS of RAS mutant cases was much lower than that of the RAS WT cases and the findings are consistent with that of the survival reported in the literature.^{8,9}

Recently, much attention has been given to the role of PIK3CA gene mutations in several human tumors. PIK3CA mutations predominantly occur in the exons 9 and 20, affecting the two functionally important helical and kinase domains of the protein.¹⁰ We observed PIK3CA mutations in 5.7% (exon 9–4.7%; exon 20–1%) cases. A few cases in our cohort presented with a synonymous p.T1025= (c.3075C > T) mutation among

other mutations. This SNV has been previously reported in ovarian, breast, and CRC (53,54). However, its clinical significance, if any, is not yet known. Different population-based studies evaluating *PIK3CA* gene mutations in CRC patients observed mutations in 9 to 16% of cases.^{11–14} A review by Li et al highlights the role of *PIK3CA* and *PTEN* alterations in the resistance pathway to anti-epidermal growth factor therapies.¹⁵ Previous studies found no prognostic significance of *PIK3CA* mutations on PFS and OS.^{16,17} A study to evaluate CRC behavior based on the exon-specific location of *PIK3CA* mutation (exon 9 or 20) reported that coexistent exon 9 and 20 mutations, but not either mutation alone can predict poor prognosis in CRC patients.¹⁸ Our results are consistent with this study as there was no case of coexistent mutations of both exon 9 and 20 observed and the individual mutations were not found to be prognostic in our study.

There are a few limitations of our study, notably, inclusion of patients of various disease stages, platforms used for mutation testing, and its sensitivity. The Cancer Genome Atlas (TCGA) study has explored the genomic as well as the transcriptomic profile of CRC; however, the pattern of occurrence of CRC and its histological variations combined with molecular profiles are varied across different populations. There can be various genetic, epigenetic, geographical, and regional variations. These factors are likely to impact the prognostic and therapeutic implications of this disease.

Conclusions

The present study helps in understanding the molecular profile of CRC cases in India. There was lower incidence of BRAF mutations in this cohort and *PIK3CA* mutation (single) did not impact survival of the patients.

Author's Contributions

Omshree Shetty and Vikas Ostwal conceptualized and designed the manuscript.

Vikas Ostwal, Anant Ramaswamy, Prabhat Bhargava, Sujay Srinivas, Avanish P Saklani, and Ashwin Desouza were involved in provision of patients.

Omshree Shetty, Vaibhavi, Akhil Kapoor, and Mukta Ramadwar were involved in collection and assembly of data. Akhil Kapoor, Omshree Shetty, Vaibhavi, Vikas Ostwal, Anant Ramaswamy, Vishakha Kamble, and Mamta Gurav were involved in data analysis and interpretation. All the authors gave final approval of the manuscript and were accountable for all aspects of the work.

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Conflicts of Interest

Nil.

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