

Clinicopathologic profile and comparative performance of limited next-generation sequencing and quantitative/real-time polymerase chain reaction for detection of RAS/RAF mutations in metastatic colorectal cancer

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Research Article

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Abstract

Background:

The implications of limited access to next-generation sequencing (NGS) relative to quantitative polymerase chain reaction (qPCR) in metastatic colorectal cancer (mCRC) remain unclear in resource-constrained settings. Our study explores correlations between genetic alterations and patient demographics and compares the sensitivity, specificity, and concordance rates of NGS-detected *KRAS*, *NRAS*, and *BRAF* mutation analyses with those detected by qPCR in mCRC.

Methods:

A prospective analysis was conducted on mCRC patients at a tertiary academic institute in India, from September 2023 to March 2024. Data on demography, pathological features, and mutations in *TP53*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, and *HER2*, detected by NGS on FFPE tumour tissue blocks, were collected. Post-NGS, adequate tumour tissue containing paraffin blocks underwent qPCR analysis of *KRAS*, *NRAS*, and *BRAF* mutations. Statistical analysis was done using SPSS Statistics (version 27).

Results:

A total of 90 patients (male: 50, female: 40; median age: 51 years) were included in the study. The majority had left-sided tumours (63.3%), with the rectum being the most common site (33.3%). The most common mutation was *TP53* [more in left-sided tumours ($p = 0.042$)]. *BRAF* mutations were more common in males ($p = 0.034$) and grade 3 histology ($p = 0.006$). Younger patients (15–39 years) had high-grade tumours ($p = 0.007$), *KRAS* mutations ($p = 0.084$), and dMMR status ($p = 0.047$). Only 39 patients were evaluable for *BRAF* mutation by qPCR after NGS, with sensitivity, specificity, and concordance of 100%, 97.29% & 97.43%, respectively. *KRAS* and *NRAS* mutations were evaluable by qPCR after NGS in 34 and 28 patients, respectively. The sensitivity, specificity, and concordance rate for *KRAS* detection were 100%, 87.5% & 85.29%, and for *NRAS*, 100%, 96.29% & 96.42%, respectively. The marginally lower specificity for *KRAS* and *NRAS* was attributable to additional variants (*BRAF* D594G; *KRAS* G13C, G12V, R68M; *NRAS* G12D) identified by NGS.

Conclusions:

Our findings provide important evidence that qPCR can identify key actionable mutations required for treatment decisions in mCRC. Performing stepwise limited NGS or qPCR initially, followed by comprehensive NGS only in highly selected patients, can optimise resource utilisation and expand equitable access to precision oncology.

Background:

Colorectal cancer (CRC) is the 3rd leading malignancy globally & is more common in the elderly age group and in males.[1] CRC accounts for about 4% in incidence as well as cancer-related mortality in India.[1] In terms of CRC-related mortality, India stands at the fifth position globally and records the 2nd largest number of rectal cancer deaths worldwide after China.[1] CRC shows a rising incidence at a younger age in the Indian population (50–69 years) as compared to the Western population (> 65 years). In India, adenocarcinomas account for nearly 93% of all CRCs based on histological evaluation and about 45% of colon cancer and 55% of rectal cancer were diagnosed with disease confined to the locoregional extent.[1]

Microsatellites are short tandemly repeated nucleotide sequences within the human genome that are susceptible to mutations, deletions & insertions in DNA coding regions, resulting in truncated proteins.[2] Mismatch repair (MMR), consisting of MLH1, MSH2, MSH6, and PMS2 proteins, corrects the errors that occur in microsatellites.[2] Failure to express one or more of these proteins culminates in mismatch repair deficiency (dMMR), whereas intact expression denotes proficient mismatch repair (pMMR) status.[2] The signalling pathway of RAS-RAF-MAPK, MSI status, proto-oncogene mutations, genes responsible for tumour suppression and the DNA MMR pathway play an important role in the development of CRC.[2]

KRAS is mutated in 35–45% of CRC cases, with the most common mutations occurring at codons 12, 13, 59, 61, and 146.[3] *NRAS* mutations are found in 5% of all CRC cases and usually do not coexist with *KRAS* mutations.[3] Approximately 10% of CRC cases exhibit the *BRAF* V600E mutation, which is found more in females, particularly in the proximal colon.[3] The *TP53* mutation, responsible for DNA damage control and intrinsic cell death mechanisms, is found in approximately 50% CRC cases, with the most commonly occurring mutations located in exons 5 and 8, specifically at codons 175, 213, 245, 248, 273, and 282.[3] The *PIK3CA* is mutated in around 10 to 20% CRC cases, of which approximately 80% of mutations are more common in the proximal colon and are localised to the hotspot regions of exons 9 and 20.[3] The *PIK3CA* mutations, particularly in exon 20, are linked with poor clinical outcomes due to negative responses predicted with anti-EGFR monoclonal antibodies.[4]

TP53 mutations lead to resistance to classical chemotherapy, but they also pave the way for immunotherapy, as such tumours are enriched for neoantigens.[5] In CRC with liver metastasis, *TP53* ranks among the most frequently mutated genes, which is also a marker of inferior overall survival.[5] *HER2* overexpression is seen in about 2% of all CRC cases and is reported in about 5–6% of *KRAS* wild-type cases in CRC.[6] *HER2* amplification or overexpression was originally recognised as a negative predictive biomarker in CRC due to its association with reduced efficacy of anti-EGFR therapies; it acts as a target for anti-*HER2* treatment in later lines.[6] This study was conducted to capture the clinicopathologic profile and comparative performance of limited next-generation sequencing (NGS) and quantitative/real-time polymerase chain reaction (qPCR) for the detection of *RAS/RAF* mutations in metastatic colorectal cancer (mCRC).

Methods:

Study Design and Patients

It was a single-centre cross-sectional study conducted at a tertiary academic institute in India. Ninety consecutive patients diagnosed with mCRC between September 2023 and March 2024 were prospectively enrolled. All histologically confirmed cases of mCRC were included. Exclusion criteria were patient age below 18 years, explicit refusal of consent, or insufficient tumour tissue for molecular analyses.

Tissue Processing and Molecular Analysis

Tumour tissue, obtained from either the primary tumour site or metastatic lesions, was processed and maintained as formalin-fixed, paraffin-embedded (FFPE) blocks. For Next-Generation Sequencing (NGS), DNA and mRNA were isolated from the FFPE tissue samples. Library construction was executed using AmpliSeq technology, followed by sequencing on the Ion S5 System. The analysis specifically targeted the *KRAS*, *NRAS*, *BRAF*, *ERBB2* (*HER2*), *PIK3CA*, *TP53*, and *NTRK* genes. A minimum sequencing depth of 500x was required for acceptance, and subsequent variant calling was performed utilising the Torrent Variant Caller and ION Reporter software. For qPCR, DNA was extracted from FFPE samples. A qPCR assay was performed utilising the Quant Studio 5 system to detect canonical hotspot mutations in the *KRAS*, *NRAS*, and *BRAF* genes. Due to inadequate tissue volume within certain FFPE blocks following NGS, paired qPCR analysis was successfully completed only on a defined subset of patients (n = 39 for *BRAF*, n = 34 for *KRAS*, n = 28 for *NRAS*).

Statistical Analysis

The collected data on age, gender, tumour grade, tumour location, mismatch repair (MMR) status by immunohistochemistry, and mutations in *TP53*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, and *HER2*, detected by NGS on formalin-fixed, paraffin-embedded tumour tissue blocks, were entered into an Excel spreadsheet. Post-NGS paraffin blocks containing enough tumour tissue remaining were taken up for qPCR analysis of *KRAS*, *NRAS*, and *BRAF* mutations, and results were evaluated using SPSS software version 27. For continuous variables, data are presented in the form of mean with the corresponding standard deviation (SD). For categorical variables, data were reported as numbers (n) and percentages (%). To determine the association between two categorical variables, the Pearson's chi-square test was employed. Differences were considered statistically significant when $p < 0.05$. Statistical analyses employed two-tailed tests, and significance was determined at $p < 0.05$. Considering qPCR as the gold standard, the sensitivity, specificity and concordance rate of NGS were compared to qPCR findings.

Results:

A total of 90 patients were enrolled in the study (Table 1), with a median age of 51 years. The majority of the patients were males, accounting for 55.6%, and left-sided tumours (63.3%) were more common than the right. The rectum was the predominant site of the primary tumour, accounting for 33.3 %. The majority of patients presented with grade 2 tumours, accounting for 40.0%. Tumours with pMMR status were present in 93.33%, while only 6.67% patients presented with dMMR tumours. The most common mutation in the study population was *TP53* (Table 2), seen in 41.1% of patients, followed by *KRAS* in 32.2%, *PIK3CA* in 17.7%, *BRAF* in 10%, and *HER2* in 2.2%.

Younger patients (15–39 years) had a higher proportion of high-grade tumours ($p = 0.007$), *KRAS* mutations ($p = 0.084$), and deficient dMMR status ($p = 0.047$) (Table 3). Females were associated with more dMMR tumours ($p = 0.047$) (Table 4), whereas *BRAF* mutations were more common in males ($p = 0.034$) (Table 5). Grade 3 histology ($p = 0.006$) (Table 5) and *TP53* Mutation were more common in left-sided tumours ($p = 0.042$) (Table 6). Only 39 patients were eligible for evaluation of *BRAF* mutation by qPCR post-NGS, with sensitivity, specificity, and concordance rates of 100%, 97.29% & 97.43%, respectively. Thirty-four and 28 patients were eligible for evaluation of *KRAS* and *NRAS* mutations by qPCR post-NGS, respectively. The sensitivity, specificity, and concordance rate for *KRAS* and *NRAS* detection were 100%, 87.5% & 85.29%, while for *NRAS*, they were 100%, 96.29% & 96.42%, respectively (Table 7).

Discussion:

This study provides valuable insights into the clinicopathological characteristics, treatment patterns and mutation profile of mCRC patients in an Indian tertiary care setting. The median age of 51 years observed in our cohort aligns with previous Indian studies, which also report a younger age of onset when compared with the Western population. For instance, a study from eastern India reported the mean age of 47 years among CRC patients, highlighting the earlier age of presentation in Indian patients.[7, 8] The majority of the patients, i.e. 55.6% were male, which is consistent with the national data. The GLOBOCAN 2022 data for India reported 40,430 cases among males and 24,433 among females, reflecting a higher incidence among males.[1] The predominance of left-sided tumours (63.3%) and rectal involvement (33.3%) in our study reflects patterns reported in other Indian studies, where rectal cancers are more prevalent.[1]

Most patients presented with moderately differentiated tumours (40%), which is in line with global histopathological distributions of CRC and with a study from a tertiary centre in India, which also reported that 47.6% of CRC cases were moderately differentiated.[7] Mismatch repair status assessment revealed that 93.33% of patients were proficient (pMMR), while 6.67% exhibited deficient MMR (dMMR). This distribution is comparable to findings from another Indian study, in which dMMR was observed in approximately 16.67% of CRC cases across all stages.[9] However, our study population included only mCRC, which may be a reason for having a lower proportion of patients with dMMR. The mutational

profile observed in the present study offers valuable insight into the molecular landscape of CRC in the Indian population and highlights differences compared with data from the Western world.

The *TP53* gene mutation was the most prevalent, identified in 41.1% of patients, aligning with global and Indian data.[3, 10] Mutations in *KRAS* were detected in 32.20 % of or population, which was lower than that of Jauhri et al. (35.7%) and higher than the retrospective study of Shetty et al. (15.4%). Since both studies included patients across all stages of CRC, whereas our study was limited to mCRC, this may explain the observed heterogeneity.[3, 11] *BRAF* mutations were found in 10% of the study population, which aligns with the prevalence reported in Western populations.[12] *NRAS* mutations were found in 5.5% of our patients, which is close to the 4% prevalence reported in the Indian study by Shetty et al., and 6.1% reported by Jauhri et al.[3, 11] In this study, we found that dMMR CRC is more common in females, consistent with previous reports.[13, 14]

Younger patients (15–39 years) had higher-grade tumours and deficient MMR (dMMR) in our study, which is consistent with previous reports describing the aggressive clinicopathological features of early-onset CRC. The higher prevalence of dMMR in younger patients is also consistent with the greater likelihood of hereditary mismatch repair deficiency in this age group.[15] *BRAF* mutations were significantly associated with grade 3 histology in our study, which is in line with previous reports showing aggressive clinicopathological characteristics, including poor differentiation.[16] However, *BRAF* mutation was more common in males in our study, which is inconsistent with previous studies, and this may be due to a limited sample size.[17] The higher prevalence of *TP53* mutations in left-sided tumours observed in our study is consistent with previous genomic studies demonstrating that TP53 alterations are enriched in left-sided CRCs.[16, 18]

In this study, we have compared NGS with qPCR for detecting *BRAF* and *KRAS* mutations in formalin-fixed, paraffin-embedded (FFPE) blocks from mCRC. For *BRAF* mutation detection, NGS showed 100% sensitivity and 97.3% specificity, with a concordance rate of 97.4% compared to qPCR, higher than the 87.8% reported by Su et al.[19] One novel variant *BRAF* D594G was additionally picked up by the NGS in one patient, and as it was not picked up by the qPCR, its inclusion in the analysis has reduced the specificity. For *KRAS* mutation detection, NGS also exhibited 100% sensitivity but a slightly lower specificity of 87.5%, with a concordance rate of 85.3% compared to qPCR, which is lower than the 92.7% reported by Su et al.[19] The NGS picked up additional mutations in 3 patients, which were G13C, G12V, and R68M. These were not detected by qPCR, resulting in reduced specificity and concordance rates.

As this was a single-center study with a modest sample size, the findings may not be readily generalizable to the wider population. Paired qPCR analysis could be performed only on a small subset of the study population due to insufficient residual FFPE tissue after NGS testing, thereby reducing the sample size available for concordance analysis. Moreover, these study findings need to be validated in a larger population with adequate tumour tissue to run both tests. Despite these limitations, the prospective design, comprehensive molecular profiling, and direct comparison between NGS and qPCR

provide clinically meaningful evidence supporting a resource-adapted molecular testing strategy for mCRC in low- and middle-income countries.

Conclusion:

In low-and middle-income country settings, where access to comprehensive NGS remains limited by cost and infrastructure, our findings provide important evidence that targeted qPCR can reliably identify key actionable mutations required for standard-of-care treatment decisions in mCRC. A stepwise testing strategy, employing limited NGS or qPCR initially followed by comprehensive NGS only in highly selected patients, can optimise resource utilisation, reduce diagnostic delays, and expand equitable access to precision oncology.

Abbreviations

RAS

Rat sarcoma virus

RAF

Rapidly accelerated fibrosarcoma

NGS

Next-generation sequencing

qPCR

quantitative polymerase chain reaction

mCRC

metastatic colorectal cancer

KRAS

Kirsten rat sarcoma viral oncogene homolog

NRAS

Neuroblastoma rat sarcoma viral oncogene homolog

BRAF

B-Raf proto-oncogene, **qPCR**:Quantitative polymerase chain reaction (also called real-time PCR)

TP53

Tumour protein p53

PIK3CA

Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic Subunit Alpha

HER2

Human epidermal growth factor receptor 2

FFPE

Formalin-fixed paraffin-embedded

SPSS

Statistical Package for the Social Sciences

dMMR

Deficient mismatch repair

BRAF D594G

Genetic mutation where the amino acid Aspartic acid (D) at position 594 in the BRAF protein is replaced by Glycine (G)

KRAS G13C, G12V, R68M

Genetic mutation where the amino acid glycine (G) at position 13 in the KRAS protein is replaced by cysteine (C), Genetic mutation where the amino acid glycine (G) at position 12 in the KRAS protein is replaced by valine (V), Genetic mutation where the amino acid Arginine (R) at position 68 in the KRAS protein is replaced by Methionine (M)

NRAS G12D

Genetic mutation where the amino acid glycine (G) at position 12 in the NRAS protein is replaced by aspartic acid (D)

CRC

colorectal cancer

MMR

Mismatch repair

MLH1

MutL homolog 1

MSH2

MutS homolog 2

MSH6

MutS homolog 6

PMS2

Postmeiotic segregation increased 2

pMMR

proficient Mismatch repair

RAS-RAF-MAPK

Rat Sarcoma (RAS), Rapidly Accelerated Fibrosarcoma (RAF), and Mitogen-Activated Protein Kinase (MAPK)

MSI

Microsatellite instability

BRAF V600E

Genetic mutation where the amino acid valine (V) at position 600 in the BRAF protein is replaced by glutamic acid (E)

EGFR

Epidermal growth factor receptor

ERBB2

Erb-B2 Receptor Tyrosine Kinase 2

NTRK

Neurotrophic tyrosine receptor kinase

SD

Standard deviation

Declarations

Ethics approval and consent to participate

This study was approved by the institutional review board, “INSTITUTIONAL ETHICS COMMITTEE, INSTITUTE OF MEDICAL SCIENCES (IMS) AND SUM HOSPITAL, Siksha ‘O’ Anusandhan (Deemed to be University)” (IEC/IMS.SH/SOA/2023/617; dated 10th October 2023), and patient consent was obtained. All procedures performed in our study involving human participation were in accordance with the ethical standards of the institutional and/or national research committee and in compliance with the 1964 Declaration of Helsinki and its later amendments. Consent to participate in the study and publication from all patients was informed to the institutional review board.

Consent for publication

Informed consent was obtained from all patients for both participation and the publication of their details.

Competing interests

None.

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None

Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AD. AD and SSP contributed to the writing of the original draft of the manuscript. AD, SSP, and SSM contributed to writing, review, and editing. SSP provided supervision for the study. All authors did the critical revision of the final manuscript. All authors confirm that they have not used any generative AI in the writing process. All authors read and approved the final manuscript. All authors confirm that the manuscript has not been submitted elsewhere for publication

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Data Availability

All data generated or analyzed during this study are included in this published article, and referenced articles are listed in the references section.

Tables

Tables 1-7 are available in the Supplementary Files section.

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