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Integration of Molecular Profiling with Microsatellite Instability in Metastatic Colorectal Cancers

Amrit Kaur Kaler[1*], Manoj Mulchandani[2], Imran Shaikh[2], Ankita Nikam[1], Kavya Shree[1], Yash Tiwarekar[1], Manish Jethani[2], Shruthi Deshkulakarani[3], Bijal Kulkarni[3], Nivetha Athikari[3], Shalaka Satpute[3], Ratika Agarwal[3], Dattatray Solanke[2], Mandar Nadkarni[2], Vaibhav Choudhary, Sandeep Goyle[2] and Rajesh Mistry[2]

[1]Department of Genetics and Molecular Medicine, Kokilaben Dhirubhai Ambani Hospital, Mumbai
[2]Centre of Cancer, Kokilaben Dhirubhai Ambani Hospital, Mumbai
[3]Department of Histopathology, Kokilaben Dhirubhai Ambani Hospital, Mumbai

[*]**Corresponding Author**: Amrit Kaur Kaler, Consultant, Molecular Pathology and Genomics Department of Genetics and Molecular Medicine, Kokilaben Dhirubhai Ambani Hospital, Mumbai

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Abstract

Background and aim

Despite significant advancements in early detection and treatment of colorectal cancer, 25% of patients present with metastatic stage and 50% with localized stages. A more comprehensive understanding of numerous genetic mutations in colorectal cancers (CRC) has paved the way towards prognosis and targeted treatments. The present study aimed to assess the integration of molecular testing and Microsatellite instability (MSI) in metastatic CRC.

Materials and methods

A retrospective cross-sectional study of all the 114 diagnosed cases of metastatic CRCs was conducted at KDAH from January 2019 to May 2024. Genomic DNA is subjected to target enrichment using a TruSight Tumor 15 amplicon library kit. The libraries were further sequenced on the Illumina Miseq NGS platform. Sequence data is analysed using a customized pipeline using Illumina basespace. MSI determination was done from PCR –sequencing by capillary electrophoresis.

Results
Most common mutations were TP53 (51%), followed by KRAS (26%), PIK3CA (8%). TP53 showed maximum co- mutation with KRAS mutation (25%), EGFR (10.29%) and ERBB2 amplifications (7.35%). MSI – HIGH with mutations were seen in 5 cases (4.42%). MSS with mutational landscape were seen in 57 (50.44%) cases and 38 cases (33.62%) cases of MSS showed no mutations.

Added targeted treatments could help increase the overall survival of patients from 6 months to more than 2 years.

Conclusion
Molecular testing is indispensable for the clinical management of colorectal cancer. It has fostered the development of new medicines, inspired the conduct of innovative clinical trials, and enabled personalized treatment to enhance efficacy.

Keywords
Colorectal cancer; Microsatellite instability; Molecular testing.

Introduction
Colorectal cancer (CRC) is the 2nd most prevalent cause of mortality from cancer and also the third most frequently diagnosed cancer globally, with 1.1 million cases reported annually. CRC is more prevalent in middle- to high-income countries, with the eightfold variation in incidence worldwide. Around 15% to 30% of patients have metastases at the time of presentation and 20% to 50% of patients who have initially localised disease will develop metastases [1].

The embryological origin of right-sided and left-sided CRCs is distinct, as they are derived from the midgut and hindgut, respectively. Females are more likely to develop right-sided CRCs, which frequently exhibit peritoneal metastatic dissemination. Men are more likely to develop left-sided tumours, which frequently metastasise to the liver and lungs. The disparity in the distribution of cancer-associated mutations is at least partially responsible for the distinct biologic behaviour of left- and right-sided tumours [2].

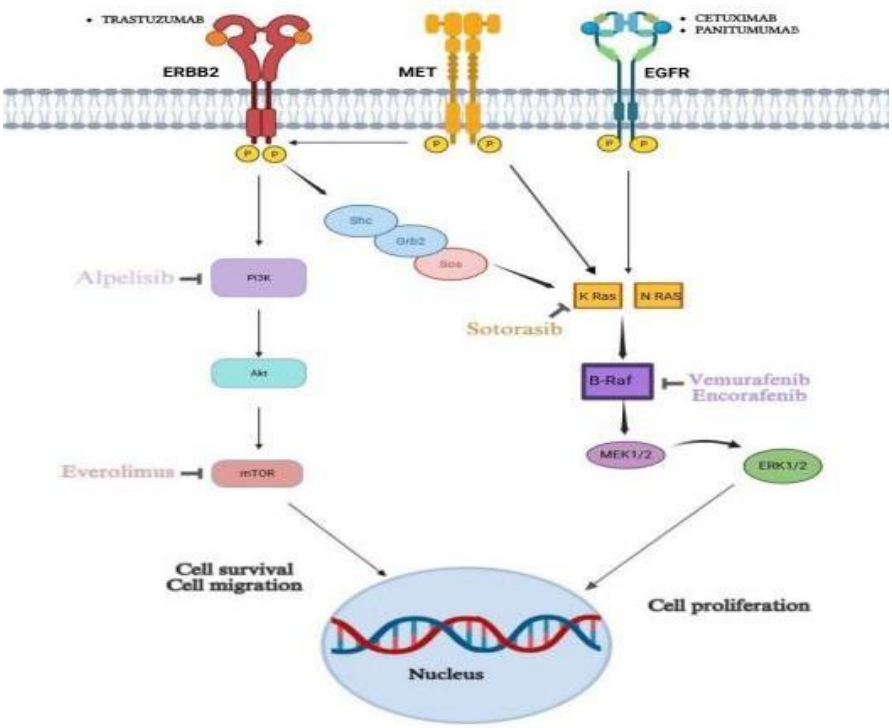
CRCs can be categorised broadly into two types: microsatellite stable tumours (MSS), which account for the overwhelming majority of CRC cases, and 10-15% of carcinomas show high-level microsatellite instability (MSI- H). Mutations in the RAS gene family are present in colorectal cancer, a disease that affects numerous molecular pathways. The CpG island methylator phenotype (CIMP) is a defining characteristic of a subset of CRCs, which is characterised by the widespread methylation of cytosine residues. BRAF has been categorized as mutation associated with Methylation of MLH1 gene. Additionally, tumour transcriptional profiles are employed to categorise CRCs. Dysregulation of the MAPK and WNT signalling pathways, chromosomal imbalances at chromosomal loci 1p, 5q, 17p, 18p, 18q, 20p, and 22q, mutations in KRAS, NRAS, or BRAF oncogenes, activation of PI3 kinase and inactivation of the TP53 gene are the most defining molecular characteristics of CRCs [3,4].

The present National Comprehensive Cancer Network (NCCN) guidelines suggest that prognostic and predictive alterations, such as KRAS, NRAS, BRAF, TP53 and PIK3CA exon20, should be considered to

inform therapeutic decision-making. Approximately 12- 15% of CRC cases exhibit microsatellite instability, which may eliminate the need for adjuvant treatment. Knowledge for classification of risks and personalized treatment options is provided by MSI testing in conjunction with molecular biomarkers.

Cetuximab and panitumumab are monoclonal antibodies that target the epidermal growth factor receptor (EGFR) and are utilised in the therapeutic management of colorectal cancer, specifically metastatic colorectal cancer (mCRC), with a particular emphasis on patients with RAS wild-type tumours [5]. Based on recent drug approvals, we designed a study to evaluate the role of extended Molecular testing with correlation of MSI in defining the pathogenesis and targeted treatment options in metastatic colorectal cancers.

Figure 1: Actionable targets in Colorectal cancers.



Materials and Methods

After obtaining clearance from ethical committee, a retrospective cross-sectional study of all the 114 diagnosed cases of metastatic CRCs was conducted in Kokilaben Dhirubhai Ambani Hospital from January 2019 to May 2024.

Inclusion criteria

- Patients with metastatic colorectal or rectal cancer with a histopathological confirmation of cancer with a diagnosis of adenocarcinoma or mucinous adenocarcinoma etc.
- Patients of all age groups.

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Exclusion criteria

- Non colorectal Cancer's tumor primaries and types
- Patients with stage T1-3 (i.e., intraepithelial, dysplasia, in situ, polyps without carcinoma).

Methodology

Genomic DNA is extracted from the formalin fixed, paraffin embedded (FFPE) tumor samples, which is subjected to target enrichment by multiplex PCR amplification using a TruSight Tumor 15 amplicon library kit. The libraries were further sequenced on the Illumina Miseq Next Generation Sequencing platform. The captured libraries are sequenced to high uniform depth (targeting >500X median coverage). Sequence data is processed using a customized analysis pipeline designed to accurately detect single nucleotide substitutions, small insertions and deletions. The reads were aligned against the whole genome build hg19 using BWA-mem. The GATK variant caller was used to detect variants at locations in the target regions. Variants were annotated using Ensemble Variant Effect Predictor. Besides, the variants were manually evaluated with COSMIC, OMIM, Varsome database.

Microsatellite status determination by PCR

Mononucleotide repeat microsatellite sequences are particularly sensitive to transcription errors, making them ideal targets for measurement by PCR amplification. To detect MSI, fluorescently labeled primers were used to amplify the target regions from the tumor and were compared with normal reference samples. The amplified fragments were subjected to capillary electrophoresis, resulting in separation of the fragments based on their size and charge. Subsequent fluorescent labelling allowed the identification of different markers. Change in sizes indicated that there is microsatellite instability, and tumors that contain this microsatellite instability are referred to MSI-high or MSI-H. The test typically used a panel of five mononucleotide markers (e.g., BAT25, BAT26, NR21, NR24, NR27). For interpretation purposes, microsatellite instability in ≥2 loci were defined as MSI-high.

Statistical analysis

Percentages, number and mean or median values along with standard deviations (mean±SD) were meticulously calculated to provide a comprehensive overview of the data collected. For comparing categorical variables, the Chi square test was employed. A p-value of less than 0.05 was established as the threshold for statistical significance, ensuring that the findings were robust and reliable. All statistical analyses were performed using the SPSS (Statistical Package for Social Sciences) version 21.0 Statistical Analysis Software.

Results

Most common mutations found were TP53 (51%) followed by KRAS (26%), PIK3CA (8%), EGFR Amp, ERBB2 Amp (4%), MET Amp (3%) and EGFR Mut, BRAF, NRAS (2%). Among the co-occurring mutation TP53 showed maximum associations with KRAS mutation (25%), EGFR (10.29%) and ERBB2 amplifications (7.35%) (MacFarlane M, 1997). Co-occurence of PIK3CA as a co-mutation with KRAS (10.29%) was the second most frequently observed alterations in the cohort. Among the co-amplifications, ERBB2 was observed in association with KRAS (5.88%) (Table 1, Figure 2).

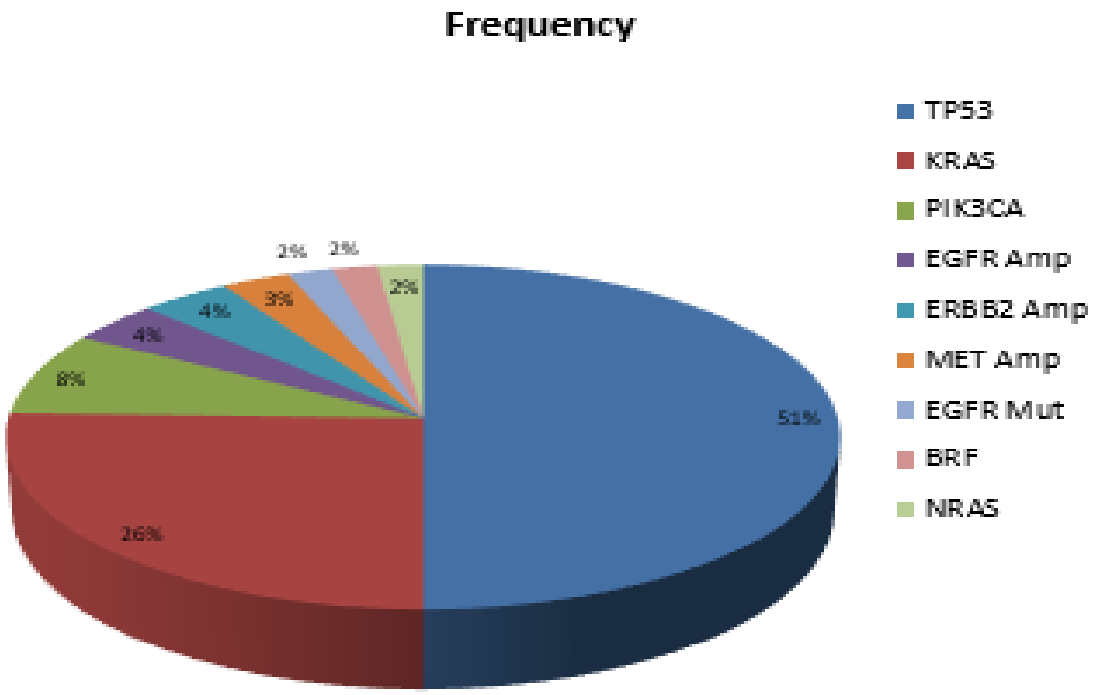


Figure 2: Distribution of gene mutations and amplifications in colorectal cancer.

Co-Existing Mutations in CRC		Driver Mutations				
		KRAS	NRAS	BRAF	EGFR Mut	ERBB2 Amplification
Co-mutations	TP53	26%	1.47%	1.47%	2.94%	7.35%
	PIK3CA	10.29%		1.47%		
	ERBB2 Amplification	5.88%	1.47%			
	AKT1	2.94%				
	MET Amplification	1.47%				
	EGFR Amplification	1.47%	2.94%			

Table 1: Co-existing mutations in colorectal cancer.

MSI –H was seen in 8.7 % of cases, while MSS was found in 83% of cases. [Figure 3] Driver mutations were seen in 5 of 10 cases (4.42%), which showed mutations in genes like EGFR, MET amp, PIK3CA and TP53 in each case, other than BRAF, hence a diagnosis of sporadic CRC was made with reduced benefit from 5-Fluoro-Uracil. Adenocarcinoma was identified in four cases, followed by mucin-secreting adenocarcinoma in one case. Mean age of the patients were 61 years and all the cases were females. Metastases were observed in the lungs, regional lymph nodes, and abdomen. The remaining 5 cases (4.42%) showed no mutations with MSI-H, hence the patients were advised germline testing to rule out hereditary predisposition. Histopathological diagnosis revealed adenocarcinoma (2 cases) and Mucin secreting (2cases), followed by signet ring cell adenocarcinoma (1case). 4 cases were found in males and 1 case was

seen in females. Metastasis was seen in lung, liver and regional lymph nodes (Table 2).

Table 3 depicted the distribution of molecular landscape in Microsatellite Stable (MSS) patients. Out of total 95 cases, mutational spectrum were seen in 57 (50.44%) cases and 38 cases (33.62%) cases showed no mutations. Most common co-mutational data is represented in table 1. Adenocarcinoma was present in 43 cases, followed by Mucin secreting (12) and signet ring cell adenocarcinoma (2). Mean age of patients was 62 years and majority of males (35) were affected than females (22). Metastasis was observed in liver, omentum, regional nodes and lungs. Hence, a diagnosis of Sporadic CRC was made in both categories, with additional targeted treatment options were recommended based on AMP and NCCN guidelines depending on mutational subtype.

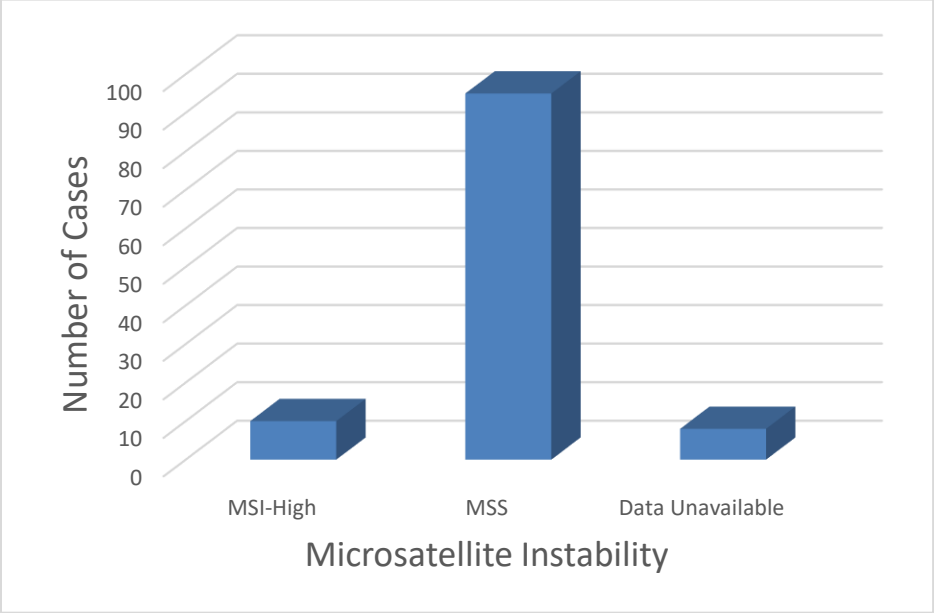


Figure 3: Prevalence of MSI Status in Colorectal Cancer Cohort.

	Microsatellite instability (MSI) – HIGH (10 cases)	
	MSI-H with mutations present	**MSI-H with no mutations**
Incidence	n = 5 (4.42%)	n = 5 (4.42%)
Common mutations	BRAF, EGFR, MET Amp, PIK3CA, TP53	None
Histopathological diagnosis	Adenocarcinoma (n=4) Mucin secreting (n=1)	Adenocarcinoma (n=4) Mucin secreting (n=1) Signet ring cell (n=1)
Mean age (years)	61	57
Gender	Female (n=5)	Male (n=4) Female (n=1)

Table 2: Distribution of MSI-H with / without mutational spectrum**.**

	Microsatellite stable (MSS) (n=95)		No data on MSI (8)
	MSS with Mutations present	**MSS with No mutations**	**Mutations present**
Incidence	n = 57 (50.44%)	n = 38 (33.62%)	n=8 (7.07%)
Common mutations	AKT1, EGFR, ERBB2, MET, PIK3CA, BRAF, KRAS, NRAS, TP53	None	KRAS, ERBB2,TP53
Histopathological diagnosis	Adenocarcinoma (n=43) Mucin secreting (n=12) Signet ring cells (n=2)	Adenocarcinoma (n=33) Mucin secreting (n=2) Signet ring cell (n=3)	Adenocarcinoma (n=6) Mucin secreting (n=1) Signet ring cell (n=1)
Mean age (years)	62	54	60
Gender	Female (n=22) Male (n=35)	Female (n=15) Male (n=23)	Female (n=5) Male (n=3)

Table 3: Distribution of Microsatellite stable (MSS) with / without mutation spectrum.

The survival data was available for limited number of patients (5 cases), for which adding targeted treatments could help increase the overall survival of patients from 6 months to more than 2 years (Table 4).

Microsatellite instability	Mutation 1	Mutation 2	Mutation 3	Overall survival
MSS	No driver mutations (n=3)	TP 53		> 2years
	KRAS G12D	ERBB2 Amp	TP53	> 2years
	KRAS G12V	PIK3CA E542K	ERBB2 H843Y	< 6 months
	PIK3CA E545K	TP53	TP53	< 6 months
	KRAS G12D			< 6 months
	KRAS G12A			< 6 months
	KRAS Q61H			< 6 months
	KRAS G12D	ERBB2 Amp	PIK3CA E545K	> 2years

Table 4: Survival Analysis (n=10).

Discussion

The emergence of CRC is a multifaceted and multistage process that involves the triggering of oncogenes and the deactivation of tumour suppressor genes [7]. It is crucial to comprehend the genomic landscape variations among different colorectal cancer subgroups in order to advance precision care [8]. Numerous studies have identified possible genetic biomarkers for CRC prognosis; however, far fewer studies have assessed markers that could predict the response to specific interventions. Numerous published studies are restricted by the limitations of early exploratory and retrospective analyses, and these biomarkers, despite their potential, have not been translated into clinical practice [9].

In the present study, the most common mutation was TP53 (51%) followed by KRAS (26%) and PIK3CA

(8%). The gene TP53 is among the most frequently mutated in human malignancies [10]. The adenoma-carcinoma transition during the tumorous pathological process is believed to be significantly influenced by p53 mutations. The TP53 mutation is present in 34% of the proximal colon tumours and in 45% of the distal colorectal tumors in CRC [11]. The majority of these mutations are located in exon 5 to 8 (DNA binding domain), with the majority occurring in certain hotspot codons, including 175, 245, 248, 273 and 282. These codons are characterized by a G to A and C to T transition, resulting in the substitution of a single amino acid in the p53 protein [12]. The disruption of specific DNA binding and sequential transactivation is most frequently the result of such substitutions clustering in the DNA binding domain. Various varieties of p53 mutations are essential in determining the biologic behavior of CRC, including the invasive depth, metastatic site, and prognosis of patients [11].

Most common TP53 co-occurring mutation showed maximum associations with KRAS mutation (26%), EGFR (10.29%) and ERBB2 amplifications (7.35%). PIK3CA was the second most common co-mutation seen in the cohort in maximum numbers with KRAS (10.29%). Among the co-amplifications, ERBB2 was seen in association with KRAS mutation (5.88%). The study conducted by Yan WF et al revealed that mutations in both TP53 (a tumor suppressor gene) and KRAS (an oncogene) are prevalent in colorectal cancers and frequently associated with poor prognosis and chemo resistance [13]. The combined mutation of both genes significantly reduces the ability to respond of colorectal cancer cells to standard first-line chemotherapy. According to Du L et al, the presence of both TP53 and KRAS mutations is associated with an increase in chemo résistance, which indicates that tumours harboring these mutations are less susceptible to conventional chemotherapy treatments [14].

It has been reported that the catalytic p110-alpha subunit of PI3K, PIK3CA, is frequently mutated in a variety of cancers, such as gastric, breast, ovarian, lung, and colorectal cancer. In two hotspots, the helicase domain of exon 9 (codons 542 and 545) and the kinase domain of exon 20 (codon 1047) over 80% of the mutations detected in PIK3CA, were reported [15]. The tumour suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a direct antagonist in the PI3K/AKT/mTOR pathway. Mutations or loss of PTEN expression have been associated with a poor prognosis in CRC [16;17]. Research has demonstrated that colorectal malignancies with both PIK3CA and KRAS mutations demonstrate severe clinicopathological characteristics, such as an increased risk of liver metastasis [18].

Luo Q et al proposed that the co-activation of MAPK and PI3K signalling pathways may be represented by KRAS and PIK3CA co-mutations [18]. Consequently, it is conceivable that the combination of KRAS and PIK3CA mutations may have mutual or additive effects on the survival of CRC patients.

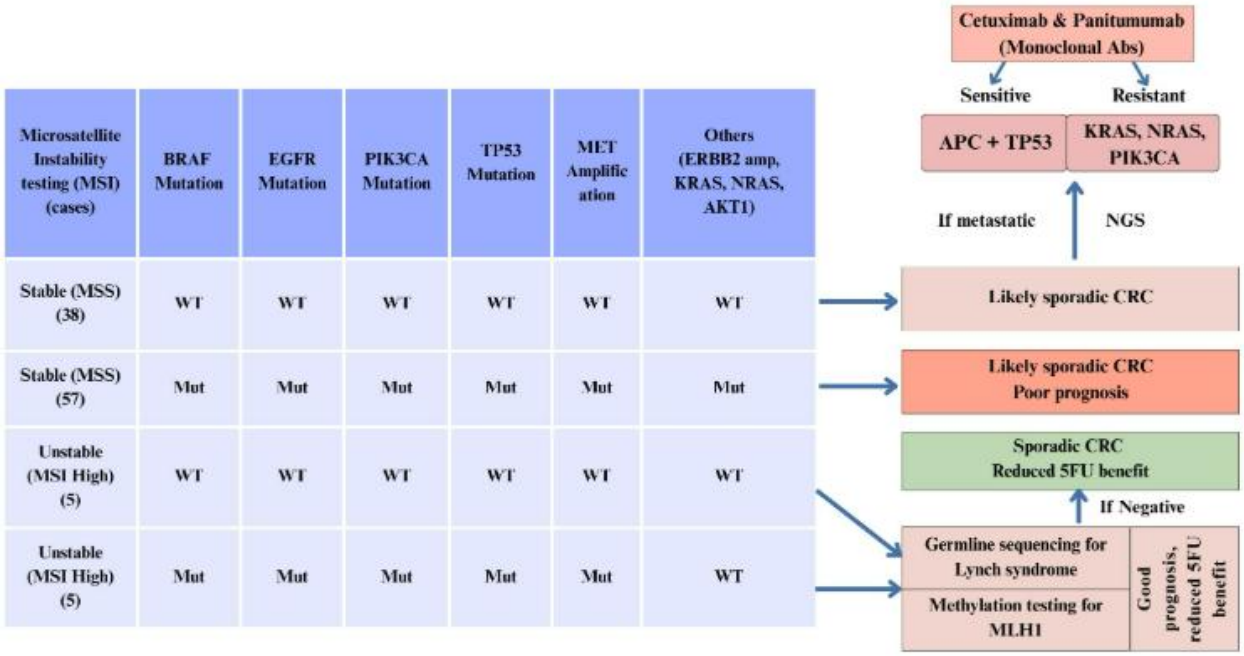
MSI is a distinctive molecular alteration that is distinguished by a high frequency of mutations in microsatellites, which are brief, repetitive DNA sequences. In early-stage CRC, MSI-H has been linked with a distinct clinical trajectory, which includes a better prognosis and an absence of advantages associated with additional therapy with 5-fluorouracil in stage II disease. In comparison to colorectal cancers with stable microsatellite instability (MSS), MSI-H CRCs demonstrate a substantially higher mutational load. The mutations recorded in this study were BRAF, EGFR, MET amp, PIK3CA, and TP53. In a study conducted by Lin EI et al, MSI-H CRCs exhibited increased rates of mutations in the BRAF, PIK3CA, and PTEN genes,

along with mutations in the receptor tyrosine kinase families [19]. In the preponderance of cases (96.2%), they recorded adenocarcinoma as the histologic diagnosis. These findings were comparable to those of our investigation. Margalef NM et al reported that MSI-H tumours in colorectal cancer, irrespective of whether they have driver mutations or not, demonstrate similar characteristics such as an increased tumour mutational burden and immune-active microenvironments [20]. This could potentially result in better outcomes and a greater responsiveness to immunotherapy.

Out of 95 MSS cases, mutations were seen in 57 cases (50.44%) and 38 cases (33.62%) showed no mutations. Most common co-mutational spectrum showed TP53 and KRAS (26%), followed by PIK3CA and KRAS (10.29%), ERBB2 and TP53 (7.35%), ERBB2amp and KRAS (5.88%) as shown in figure 3. In a study conducted by Dos Santos W et al, prevalent driver mutations are frequently observed in genes such as APC, TP53, and KRAS in microsatellite stable (MSS) colorectal cancer (CRC) [21]. It is essential to comprehend the driver mutations in MSS CRC in order to predict patient outcomes and devise targeted therapies. KRAS, NRAS, BRAF, PIK3CA, ERBB2 Amplification was the most common driver mutations but provided resistance to specific anti-EGFR therapies. The existence of specific mutations, such as those in TP53, can be linked to a worse prognosis.

Li L et al demonstrated that the transformation from healthy cells to the first malignant cell was facilitated by the variations in three driver genes, APC, KRAS, and TP53 [22]. Metastasis in patients with driver mutation was observed in the liver, omentum, regional nodes, and lungs. Driver mutations are present in both primary and metastatic tumours, suggesting that metastatic colorectal cancer can advance early during tumour development, as suggested by Huang D et al [23].

Cetuximab / panitumumab, two monoclonal antibodies (mAbs) was started in 3 patients as monotherapy or in combination with chemotherapy to treat patients with RAS wild-type metastatic colorectal cancer, with the overall survival time was increased to more than 2 years. According to Hong Xie Y et al, targeted therapy is a novel, alternative approach that has effectively extended the overall survival of patients with colorectal cancer [24]. The average duration of survival for patients with metastatic colorectal cancer (mCRC) has increased from 3.6–6 months to 24–28 months as a result of the development of molecularly targeted treatments, immune checkpoint inhibitors, and evolving surgical techniques for treating liver and lung metastatic lesions, as reported by Li F et al [25]. Cetuximab can elicit immune functions like T-cell priming through dendritic cell maturation, antibody-dependent cell-mediated cytotoxicity involving natural killer cells, and T-cell recruitment to the tumour, hence can change a cold tumor to hot. These immune functions are not possessed by panitumumab, an IgG2 isotype mAb [24].



Microsatellite Instability testing (MSI) (cases)	BRAF Mutation	EGFR Mutation	PIK3CA Mutation	TP53 Mutation	MET Amplification	Others (ERBB2 amp, KRAS, NRAS, AKT1)
Stable (MSS) (38)	WT	WT	WT	WT	WT	WT
Stable (MSS) (57)	Mut	Mut	Mut	Mut	Mut	Mut
Unstable (MSI High) (5)	WT	WT	WT	WT	WT	WT
Unstable (MSI High) (5)	Mut	Mut	Mut	Mut	Mut	WT

Figure 4: Diagnostic Interpretation after Molecular testing and MSI in metastatic colorectal cancers.

Similarly, KRAS G12D gene mutation in association with ERBB2 Amp and TP53 gene mutation resulted in good survival as the patient received Trastuzumab and Pertuzumab combination therapy. Association of KRAS G12V gene mutation with PIK3CA E542K gene and ERBB2 H843Y gene mutation leads to poor with survival less than 6 months. In a similar way, PIK3CA E545K mutation in association with two TP53 genes mutation resulted in poor survival. Various gene mutations have the potential to affect the development of tumours, immune response, and response to therapy, thereby affecting cancer biology and treatment outcomes. The current study demonstrated that the addition of targeted treatments could enhance the overall survival of patients from six months to over two years in a five patients.

This limitation was due to patient affordability, education and many patients were lost to followup. Microsatellite status has been predicted to decreased efficacy in cetuximab treatment. In CALGB/SWOG 80405 study, patients showed worse overall survival in MSI-H tumours showed with cetuximab arm rather arm bevacizumab arm [26]. 27. Zhou J et al proposed that MSI may interact with oncogenic drivers such as BRAF and ERBB2 to promote cetuximab resistance in their study [27].

Conclusion

Our results provide insight into the molecular basis of colorectal cancer and proposed the potential of sequencing for bigger panel involving molecular testing and MSI simultaneously, which can be indispensable for the clinical management of colorectal cancer. The mutational spectrum helped us understand pathogenesis, guide prognosis, prioritize the targeted treatment options with improved overall survival. It has fostered that the study of interaction of these mutations will inspire the development the conduct of innovative clinical trials, and enabled personalized treatment to enhance efficacy. The future endeavors should focus on the development of tumor microenvironment with study

on combination of multi-omics biomarkers, which can define and a more comprehensive classification of CRCs and actionability.

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