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Analyzing Colorectal Cancer at the Molecular Level through Next-generation Sequencing in Erbil City

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***Abstract*—Colorectal cancer (CRC) ranks as the third leading cause of cancer-related deaths globally. It is characterized as a genomic disorder marked by diverse genomic anomalies, including point mutations, genomic rearrangements, gene fusions, and alterations in chromosomal copy numbers. This research aims to identify previously undisclosed genetic variants associated with an increased risk of CRC by employing next-generation sequencing technology. Genomic DNA was extracted from blood specimens of five CRC patients. The sequencing data of the samples are utilized for variant identification. In addition, the Integrative Genomic Viewer software (IGV) is used to visualize the identified variants. Furthermore, various *in silico* tools, including Mutation Taster and Align GVGD, are used to predict the potential impact of mutations on structural features and protein function. Based on the findings of this research, 12 different genetic variations are detected among individuals with CRC. Inherited variations are located within the following genes: MSH6, MSH2, PTPRJ, PMS2, TP53, BRAF, APC, and PIK3CA.**

***Index Terms*—Colorectal, Cancer, Gene, Molecular level, Mutation.**

I. Introduction

Colorectal cancer (CRC) is a significant worldwide contributor to illness and health-related issues (Arbyn, et al., 2020). Globally, CRC leads to substantial sickness and loss of life. It stands as the prevalent form of internal cancer in both men and women within Western communities. The occurrence of CRC is progressively increasing in Asian countries as well. Among the genetic triggers of CRC, Lynch syndrome (LS), alternatively known as hereditary non-polyposis CRC, holds the highest prevalence, contributing to about 3–4% of all global cases of CRC (Sinicrope, 2018). Certainly, both inherited and acquired genetic alterations, along with external environmental influences, undoubtedly play a role in its development. CRC displays significant diversity in terms of tumor location, genetic variations, and racial distinctions. The interplay of various factors, including environmental elements, dietary choices, and lifestyle, exerts multiple influences on the progression of the disease(Murphy, et al., 2019). Risk factors for CRC encompass a range of factors such as genetic predisposition (like hereditary non-polyposis CRC), susceptibility to polyp development, inflammatory conditions in the large bowel, obesity, consumption of high-fat diets, alcohol consumption, smoking, and psychological stress (Murphy, et al., 2019; Tariq and Ghias, 2016; Barrasa, et al., 2013). For a considerable time, it has been acknowledged that certain families exhibit a dominant inclination toward developing colorectal adenomas and/or cancers. However, the discovery of the specific genes and mutations accountable for this heightened familial susceptibility is a relatively recent development (Peltokallio and Peltokallio, 1966; Lynch and Lynch, 1985). The risk levels elevate significantly when multiple family members are impacted or if an individual within the family is diagnosed at an early age (Fuchs, et al., 1994; Winawer, et al., 2003). The development of CRC results from the progressive buildup of genetic modifications (such as gene mutations and amplification) and epigenetic modifications including abnormal DNA methylation and changes in chromatin structure (Bardhan and Liu, 2013). These alterations collectively convert normal colonic epithelial cells into malignant colon adenocarcinoma cells. The initial stages of tumorigenesis involve the loss of genomic stability, leading to genetic changes. This process is pivotal in acquiring the necessary mutations in tumor suppressor genes and oncogenes, which drive the transformation of cells and facilitate the advancement of tumors (Kontomanolis, et al., 2020). Two primary types of genomic instability, namely, microsatellite instability and chromosome instability, have been recognized as predominant in colon cancer (Rao and Yamada, 2013). The impacts of diverse types of genomic instability on the biological and clinical characteristics of colon tumors were examined (Hause, et al., 2016). Investigating the origins and functions of genomic and epigenomic instability in the development of colon tumors holds the promise of generating improved approaches for preventing and treating CRC

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in patients. Next-generation sequencing (NGS) is a gene sequencing technology that offers very high throughput, scalability, and speed, allowing sequencing of whole cancer genomes (whole genome sequencing or WGS) from dozens to hundreds of patients within a few days. Significant variations in the lifetime susceptibility to cancer have been documented among carriers of MMR (mismatch repair) mutations, with the greatest risk associated with mutations found in either MLH1 or MSH2 genes. Cancer occurrences within families possessing a pathogenic alteration in MSH6 are typically detected at a later stage, and the associated cancer risks are relatively diminished, except in the case of endometrial cancer (Berends, et al., 2002; Hendriks, et al., 2004; Baglietto, et al., 2010). Most investigations detailing the molecular makeup of LS families have been carried out in regions encompassing North America, Europe, and Asia (Dominguez-Valentin, et al., 2013). A limited number of studies have outlined the occurrence and specific varieties of MMR (mismatch repair) mutations in Latin America, particularly within the context of Brazil (Cossio, et al., 2010; Da Silva, et al., 2010; Carneiro Da Silva, et al., 2015).

The objective of this research was to assess the effectiveness of next-generation sequencing (NGS) in a standard clinical environment involving patients with CRC. Exome sequencing was employed to reveal previously unidentified variants that predispose individuals to CRC.

II. Materials and Methods

A. Collection of Samples

The present study included five CRC patients recruited from Rezgari Hospital, Erbil/Iraq. A case–control study design was established from (August 01, 2022, to December 01, 2022) to study the effect of some genetic effects in patients with CRC who visited Rezgari Hospital Cancer Department, Erbil/Iraq.

B. Extraction of Genome

Genomic DNA from blood specimens was prepared using a DNA extraction kit (Thermofisher, USA), following the manufacturer's instructions with minor modifications. Qualification and quantification of DNA concentration were performed using NanoDrop (Biometrical). Samples of genomic DNA with (A260–A230)/(A280–A320) ratios of more than 1.7 and outputs more than 30 ng/µl were obtained.

C. Mutation Analysis

Twist Human Core Exome Enzymatic Fragmentation (EF) Multiplex Complete kit was used for library construction, and MGIEasy FS DNA Library Prep Kit was performed for the library to be ready for sequencing on the MGI system. The library was sequenced on the (MGI-DNBSEQ-G400, China) instrument generating 150 bp paired-end read with 100X mean target coverage. Fast QC, and Raw fastq files were quality controlled. The reads were aligned to the reference human genome (hg19) using Burrows-Wheeler Aligner (BWA). Variants were identified with the Genome Analysis Toolkit (GATK). Integrative Genomic Viewer software (IGV) was used for variants visualization Intergen Genetics and Rare Diseases Diagnostic Center, (Ankara, Turkey).

D. In Silico Analysis

Different *in silico* tools were used to predict the effect of mutation on the structural features or protein function. Polymorphism Phenotyping (PolyPhen-2) (Vaser, et al., 2016) and Sorting Intolerant from Tolerant (SIFT) (Schwarz, et al., 2014) were used to assess the functional effects of variants. A mutation Taster was used for the evaluation of the mutation effect on protein function and structure (Tavtigian, et al., 2006). Align GVGD was used to compute a biochemical distance score prepared manuscript methods according to previous international published NGS data. In the thesis, we explained and clarified the method section in detail (Tavtigian, et al., 2006; Mathe, et al., 2006).

III. Results and Discussion

In this study, NGS was employed for the exploration of the genetic mutation landscape within a cohort of five CRC patients residing in Erbil City/Iraq. Previously, immunohistochemical and molecular studies were conducted to target the expression of specific genes (Kamal and Jalal, 2019; Ali Hama, 2019). In addition, other studies attempted to determine mutations in target genes among patients with CRC (Abid, Qadir and Salihi, 2021). To the best of our knowledge, this is the inaugural study conducted in Erbil City/Iraq to evaluate the use of NGS in a typical clinical setting with CRC patients. Exome sequencing was utilized to identify previously unknown genetic variations that may increase an individual's susceptibility to CRC. The 12 variants in CRC patients were identified and the inherited variants were found in MSH6, MSH2, PTPRJ, PMS2, TP53, BRAF, APC, and PIK3CA genes. Table I shows the variants and the results of *in silico* predictions.

For predicting the effects and potential significance of variants, many *in silico* tools have been developed. Polymorphism Phenotyping (PolyPhen), SIFT, and MutationTaster were applied to investigate the functional effects of 12 variants. The PolyPhen gives predictions to find structural features and sequence alignment changes caused by amino acid substitution. PolyPhen predicts the functional significance of an allele substitution. Variants with scores of 0.0 are predicted to be benign, the scores 0.15–0.85 are predicted as possibly damaging, the score more than 0.85 are more confidently considered as damaging. PolyPhen predicted uncertain significance function for a variant of MSH2, TP53, BRAF, and a variant of APC. Furthermore, it predicted probably damaging function for a variant of MHS6 (ENST00000234420.11 c.276A>G).

The functional effect of SIFT on the variants is assessed by evaluating the sequence homology and investigating the conservation degree of amino acid residues among species. SIFT predicts whether an amino acid substitution in a protein will have a phenotypic influence. A score ≤0.05 is predicted

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TABLE I
MUTATIONS IDENTIFIED IN COLORECTAL CANCER PATIENTS

Gene	Variant coordinates	Amino acid change	Zygosity	*In silico* parameters*	Type and Classification
MSH6	ENST00000234420.11 c.276A >G	p.P92P	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A Mutation Taster: N/A Conservation_nt: N/A Conservation_aa: N/A	Likely pathogenic
MSH2	47403411 C >G	Splicing site	Homozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Uncertain significance
PTPRJ	ENST00000418331.7 c.827A >C	p.Q276P	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Pathogenic
PMS2	5982995 C >T	Splicing site	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Likely pathogenic
TP53	ENST00000359597.8 c.215C >G	p.P72R	Homozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Uncertain_significance
BRAF	140734797 C>A	Intronic	Homozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Uncertain_significance
APC	ENST00000257430.9 c.5880G >A	p.P1960P	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Likely_pathogenic
APC	ENST00000257430.9 c.5034G >A	p.G1678G	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Uncertain_significance
APC	ENST00000257430.9 c.4479G >A	p.T1493T	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Uncertain_significance
APC	ENST00000504915.2 c.147T >C	p.Y49Y	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Uncertain_significance
APC	112707585 C >G	Intronic	Heterozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Uncertain_significance
PIK3CA	179199217 A >G	Intronic	Homozygote	PolyPhen: - Align-GVGD: N/A SIFT: N/A MutationTaster: N/A Conservation_nt: N/A Conservation_aa: N/A	Likely pathogenic

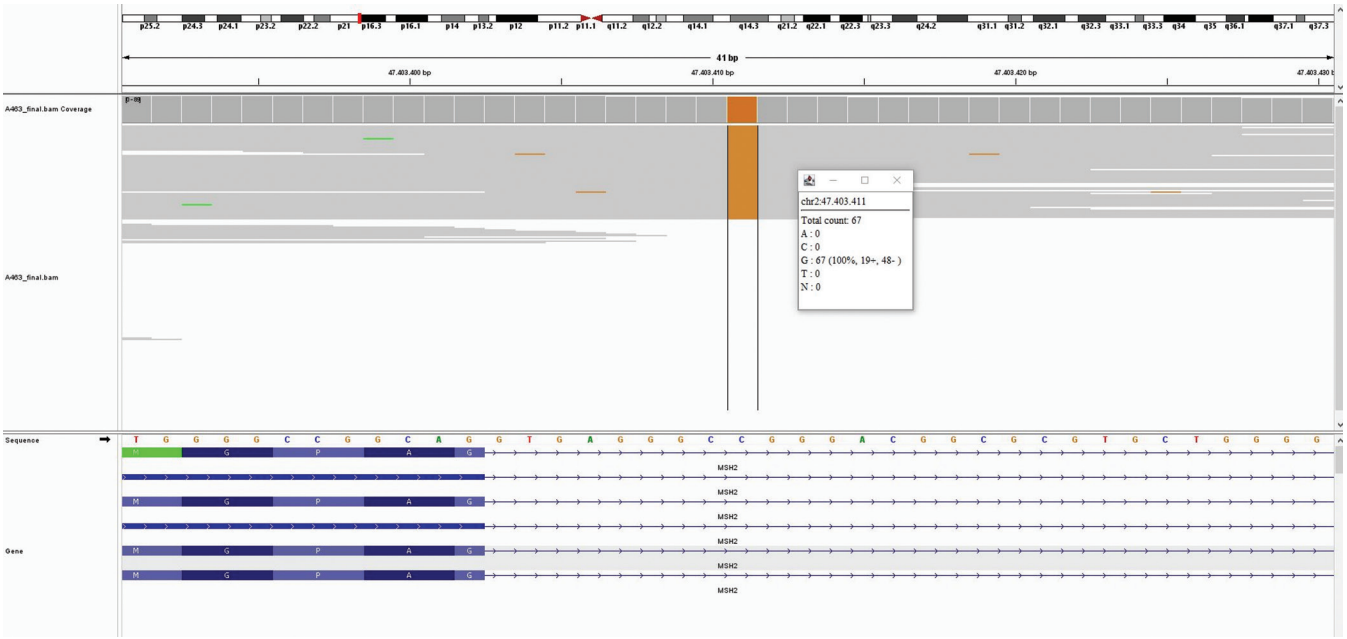

Fig. 1. Integrative Genomics Viewer image of next-generation sequencing data of MSH2 47403411 C>G variant detected.

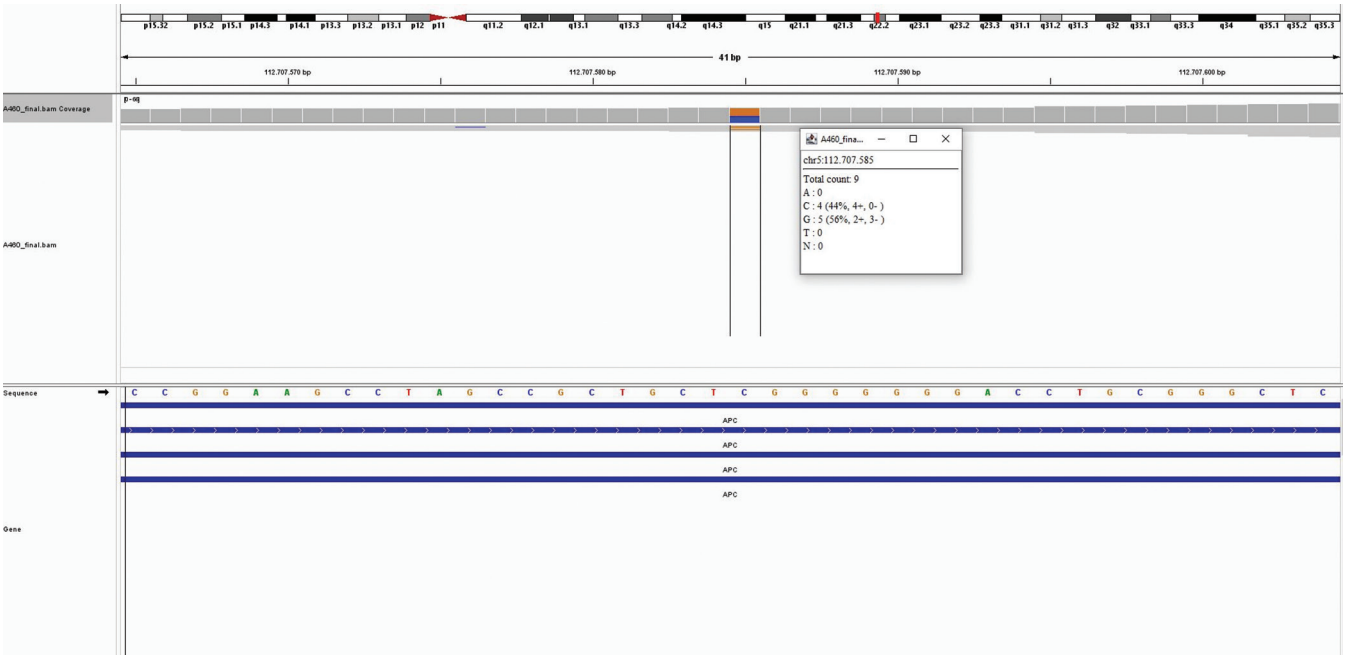

Fig. 2. Integrative Genomics Viewer image of next-generation sequencing data of Adenomatous Polyposis Coli 112707585 C>G variant detected.

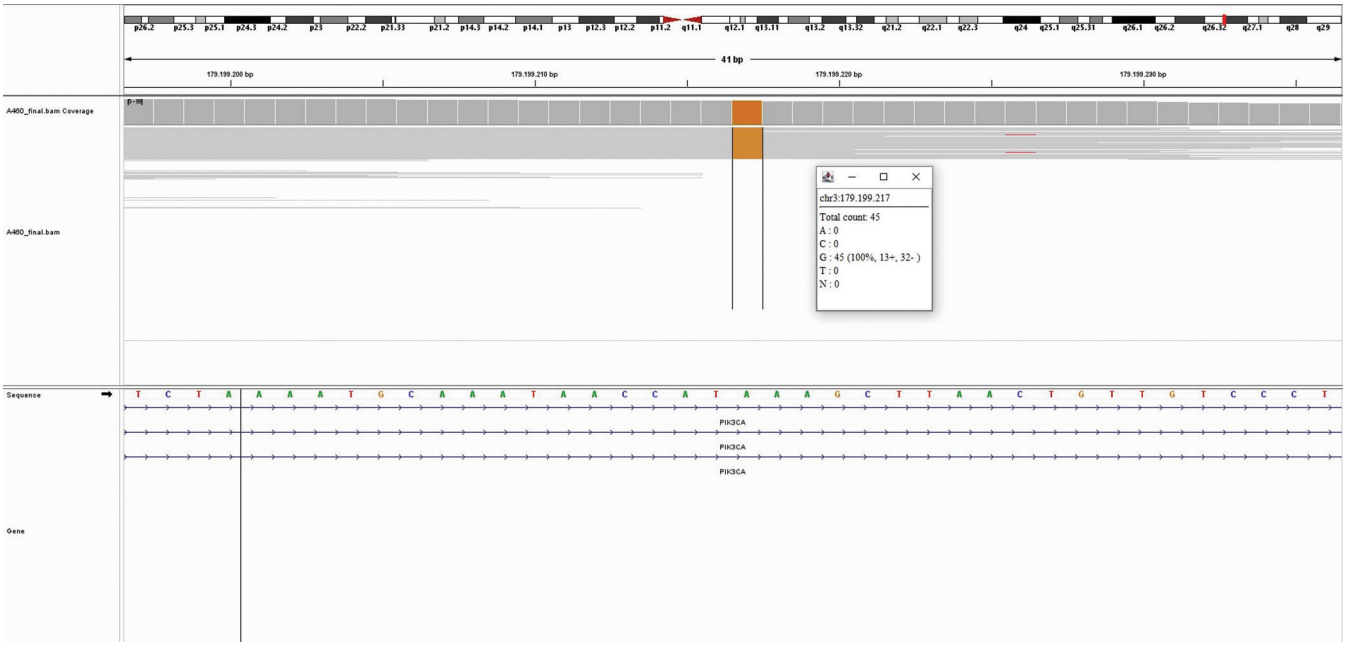

Fig. 3. Integrative Genomics Viewer image of next-generation sequencing data of PIK3CA 179199217 A>G variant detected.

to be tolerated, and a score less than 0.05 is predicted to be deleterious. SIFT predicted tolerated effects in a variant of APC and BRAF as well as the deleterious effects in a variant of MSH6, PIK3CA, and PMS2.

Mutation Taster evaluates mutation effect on protein function and structure. It considers the effect of mRNA expression or splicing (Malińska, et al., 2020). It predicts the disease potential of an alteration as disease causing which is probably deleterious, disease-causing automatic which is deleterious, polymorphism which is probably harmless, and polymorphism automatic which is harmless. The results predicted disease causing in a variant of MSH6, APC, and A variant of PIK3CA (Perne, et al., 2021). In many cases, the first mutation occurs in the APC gene. This leads to an increased growth of colorectal cells because of the loss of this "brake" on cell growth. Further mutations may then occur in other genes, which can lead the cells to grow and spread uncontrollably.

The roles of these genes were known in the literature.

- MutS Homolog 6 (MSH6): MSH6 is a crucial component of the DNA mismatch repair (MMR) system. Mutations in MSH6 can lead to microsatellite instability (MSI), a hallmark of CRC. MSI can result in the accumulation of errors during DNA replication, contributing to tumorigenesis (Boland and Goel, 2010)
- MutS Homolog 2 (MSH2): MSH2 is an essential component of the DNA mismatch repair system. Mutations in MSH2

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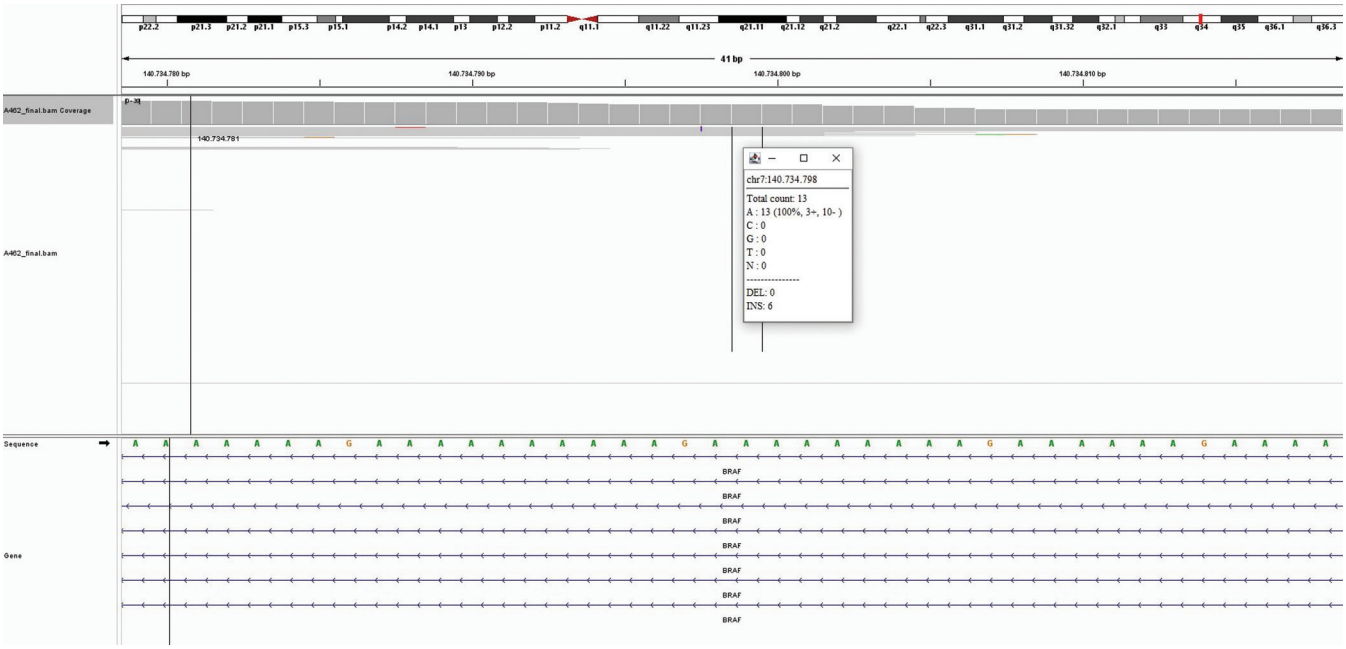

Fig. 4. Integrative Genomics Viewer image of next-generation sequencing data of BRAF 140734797 ?>A variant detected.

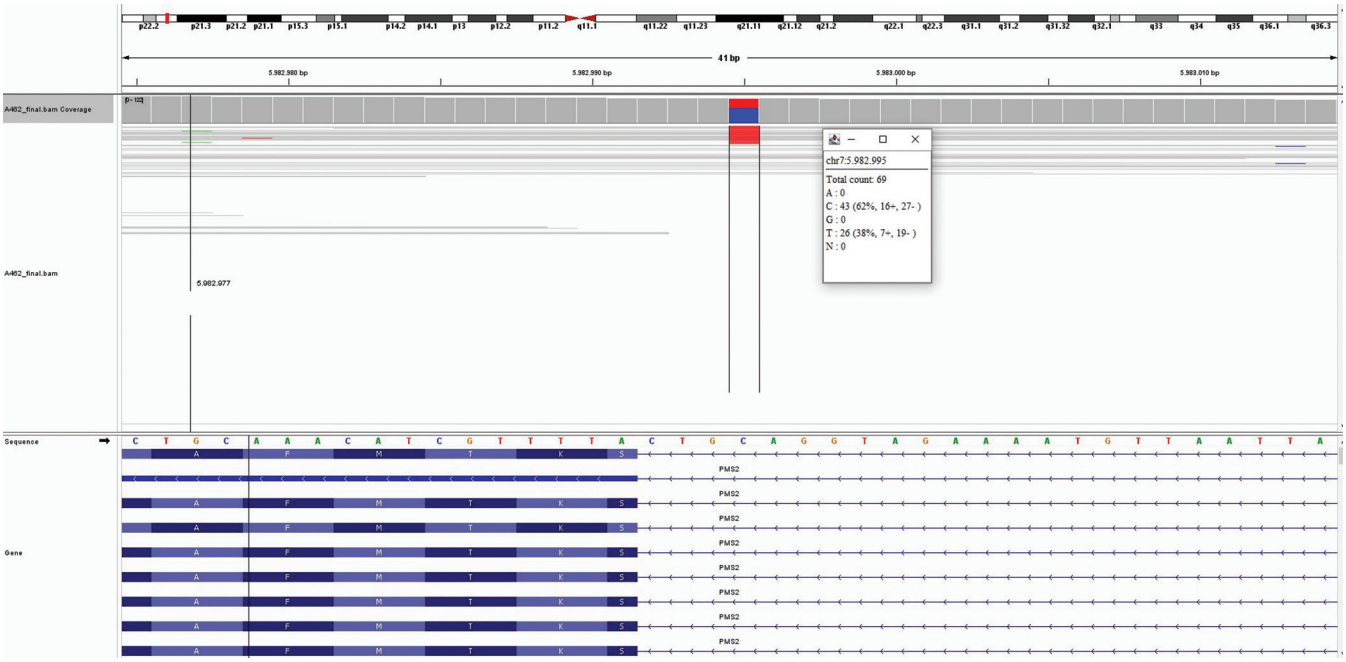

Fig. 5. Integrative Genomics Viewer image of next-generation sequencing data of PMS2 5982995 C>T variant detected.

can impair the correction of DNA replication errors, leading to MSI and an increased risk of CRC (Lynch and De la Chapelle, 1999)

- Protein Tyrosine Phosphatase, Receptor Type J (PTPRJ): PTPRJ is a receptor-type protein tyrosine phosphatase involved in cell adhesion and signaling. In CRC, mutations in PTPRJ may disrupt cellular communication and adhesion, contributing to cancer progression (Li, et al., 2022)
- PMS2 (PMS1 Homolog 2, Mismatch Repair System Component): PMS2 is a key player in the DNA mismatch repair system. Mutations in PMS2 can compromise the repair of DNA errors, leading to MSI and an increased susceptibility to CRC (Ten Broeke, et al., 2015)
- Tumor Protein P53 (TP53): TP53 is a tumor suppressor gene crucial for cell cycle regulation and DNA repair. Mutations in TP53 are common in CRC and can result in the loss of its tumor-suppressing function, leading to uncontrolled cell growth (Olivier, Hollstein and Hainaut, 2010)
- B-Raf Proto-Oncogene, Serine/Threonine Kinase (BRAF): BRAF is a proto-oncogene involved in the RAS/RAF/MEK/ERK signaling pathway. Mutations in BRAF, particularly the V600E mutation, are associated with a subset of CRCs, leading to increased cell proliferation (Davies, et al., 2002)
- Adenomatous Polyposis Coli (APC): APC is a critical tumor suppressor gene that regulates the Wnt signaling pathway. Mutations in APC are early events in colorectal

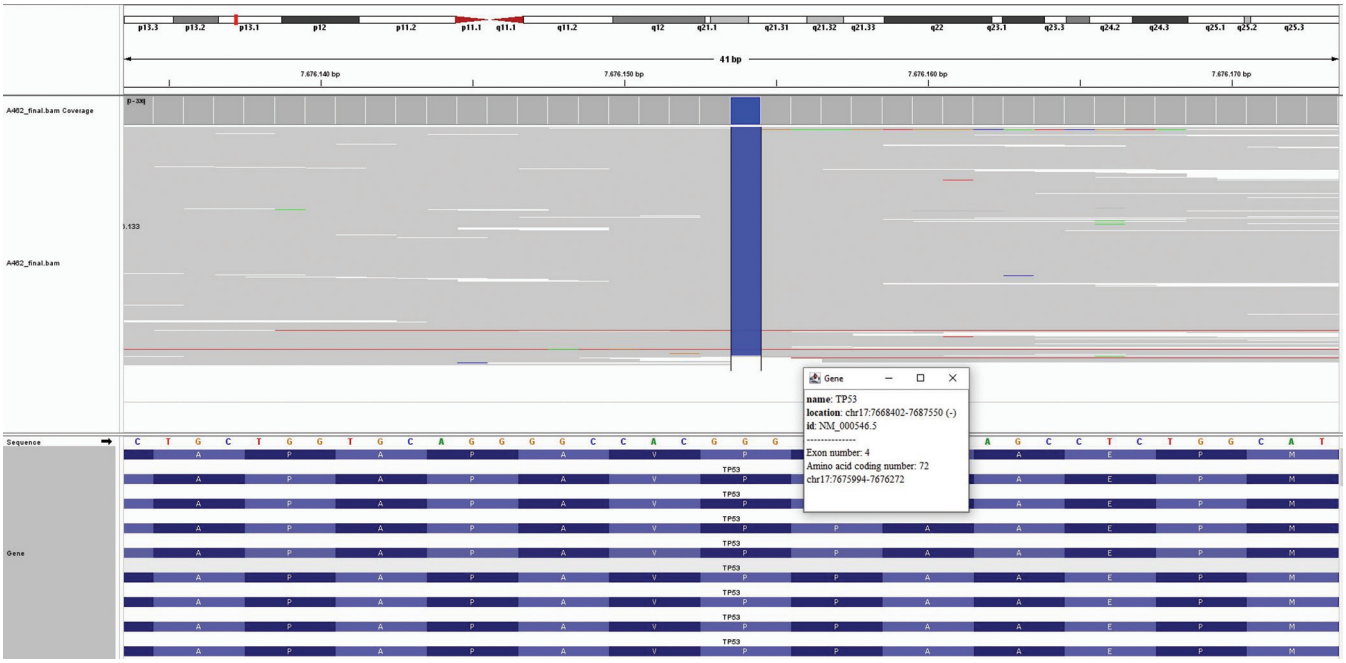

Fig. 6. Integrative Genomics Viewer image of next-generation sequencing data of TP53 ENST00000359597.8 c.215C>G variant detected.

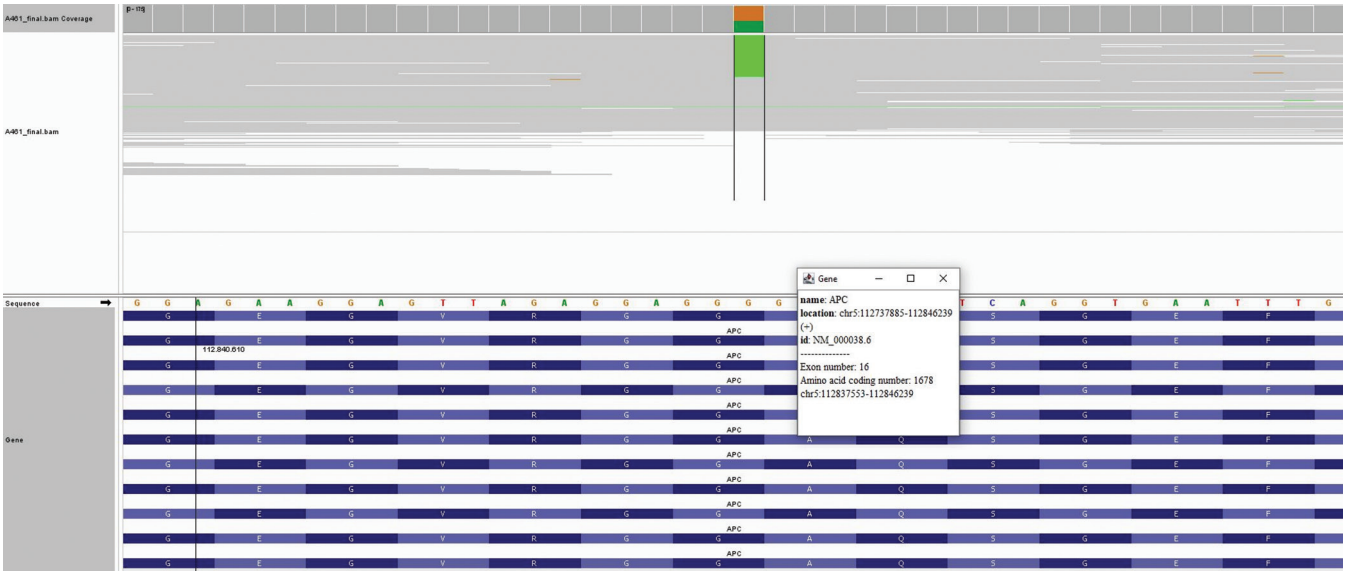

Fig. 7. Integrative Genomics Viewer image of next-generation sequencing data of Adenomatous Polyposis Coli ENST00000257430.9 c.5034G>A variant detected.

carcinogenesis, leading to uncontrolled cell proliferation and the formation of adenomas (Segditsas and Tomlinson, 2006)

- Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA): PIK3CA is involved in the PI3K/AKT/mTOR signaling pathway, regulating cell survival and growth. Mutations in PIK3CA can lead to increased PI3K activity, promoting CRC development (Janku, et al., 2012).

The initial discovery of a pathogenic MSH6 variant occurred in 1997 within a family afflicted by multiple LS-spectrum tumors, and subsequent investigations have consistently demonstrated that individuals with pathogenic MSH6 variants exhibit a reduced risk and delayed onset of colorectal cancer when compared to those with MLH1 and MSH2 variants as shown in Fig. 1, (Frederiksen, et al., 2021). For instance, when individuals with MSH6 variants reach the age of 70, they face a CRC risk of 20% for males and 12% for females. In contrast, male and female carriers of MLH1 variants have a higher risk (Dominguez-Valentin, et al., 2020).

According to the genetic analysis study of the present work, the HOMOZYGOTE splicing site was detected at 47403411 C>G which was classified as uncertain significance as shown in Fig. 2. The MSH2 variant exhibits the second-highest CRC risk, closely trailing MLH1, with an impact more pronounced

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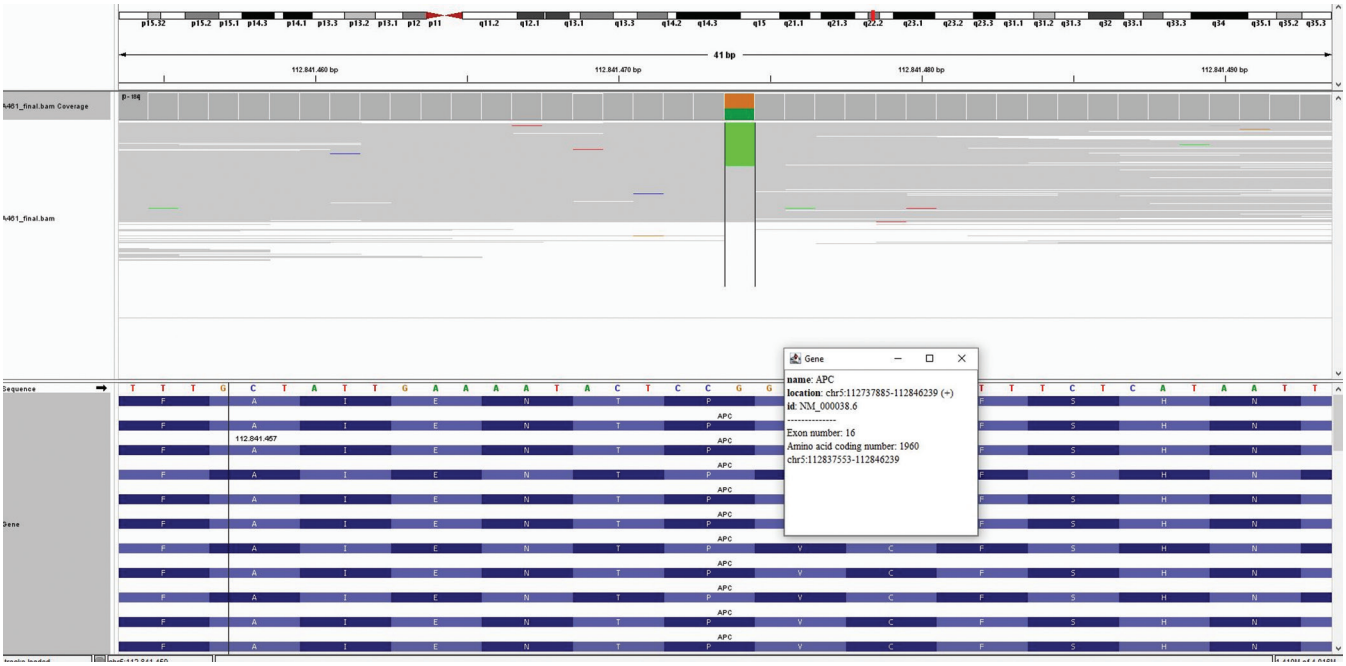

Fig. 8. Integrative Genomics Viewer image of next-generation sequencing data of Adenomatous Polyposis Coli ENST00000257430.9 c.5880G>A variant detected.

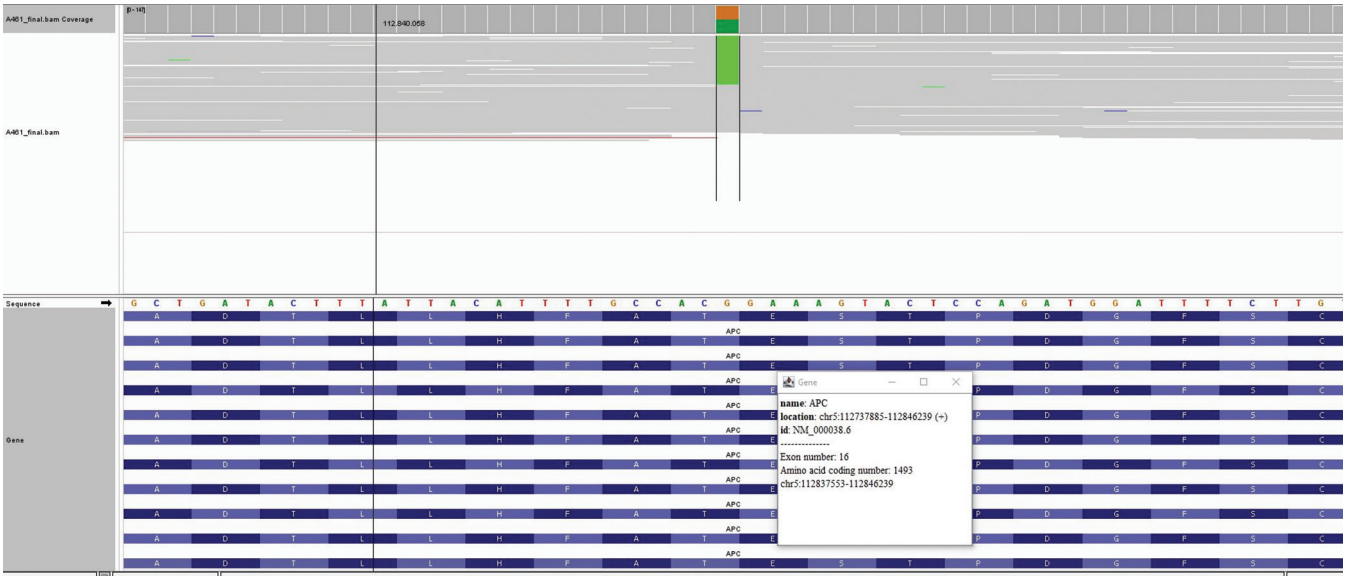

Fig. 9. Integrative Genomics Viewer image of next-generation sequencing data of Adenomatous Polyposis Coli ENST00000257430.9 c.4479G>A variant detected.

in individuals aged over 75, affecting 46.6% of women and 51.4% of men carrying this genetic variation (Dominguez-Valentin, et al., 2020; Fatemi, et al., 2023).

Our examination in Fig. 3 underscores the identification of the heterozygous variant c.827A>C within the tumor suppressor gene PTPRJ, which has been classified as having pathogenic significance. Protein tyrosine phosphatase receptor type J (PTPRJ) is a gene known for its tumor-suppressing properties, as it exerts a negative regulatory influence on critical processes like angiogenesis, cell proliferation, and migration that make it a crucial player in the context of tumor formation and development (Laczmanska and Sasiadek, 2019). A prior investigation verified that the presence of c.827A>C markedly elevates the risk of developing colon cancer (Mita, et al., 2010).

Fig. 4 shows that HETEROZYGOTE PMS2 is observed at the splicing site (5982995 C>T). Usually, PMS2 mutations are implicated in lynch syndrome (LS) associated colorectal cancer in approximately 8 to 15% of cases, with variable incidence rates and depending on diagnostic methods such as PCR, microsatellite instability, IHC, or DNA sequencing (Poaty et al., 2023). Heterozygous PMS2 mutations were identified in 90.16% (55/61) of cases, while homozygous PMS2 mutations were observed in 9.83% (6/61) of LS cases (Senter, et al., 2008).

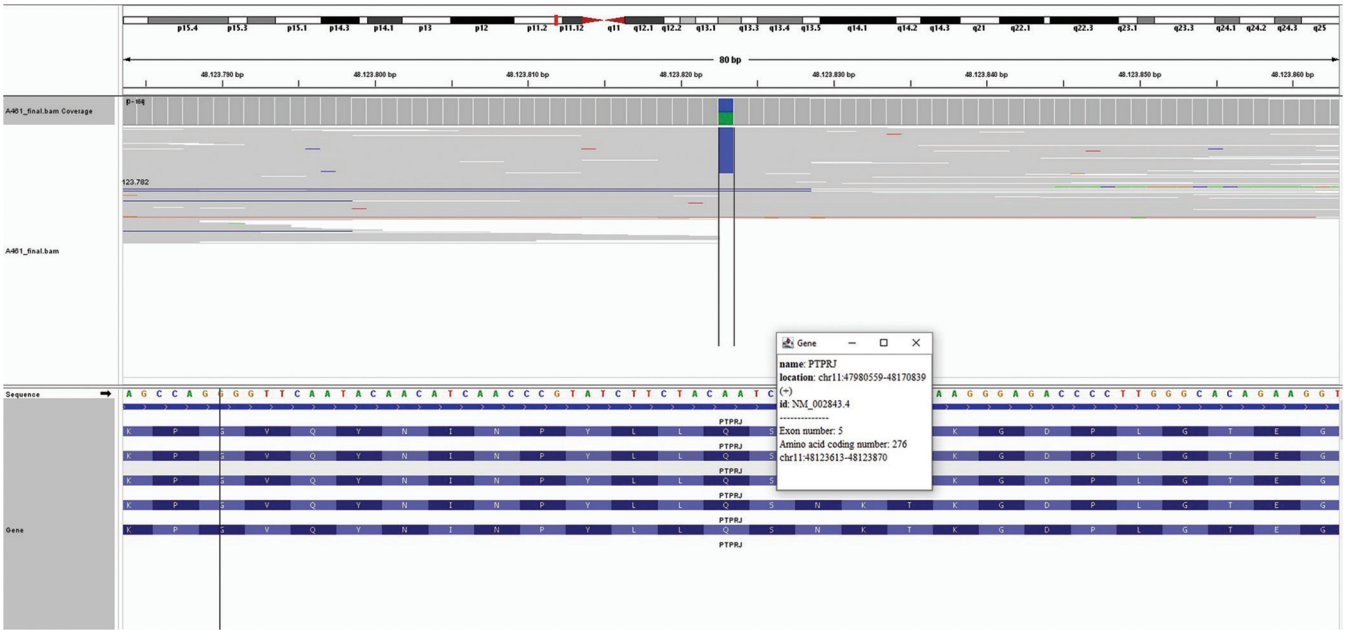

Fig. 10. Integrative Genomics Viewer image of next-generation sequencing data of PTPRJ ENST00000418331.7 c.827A>C variant detected.

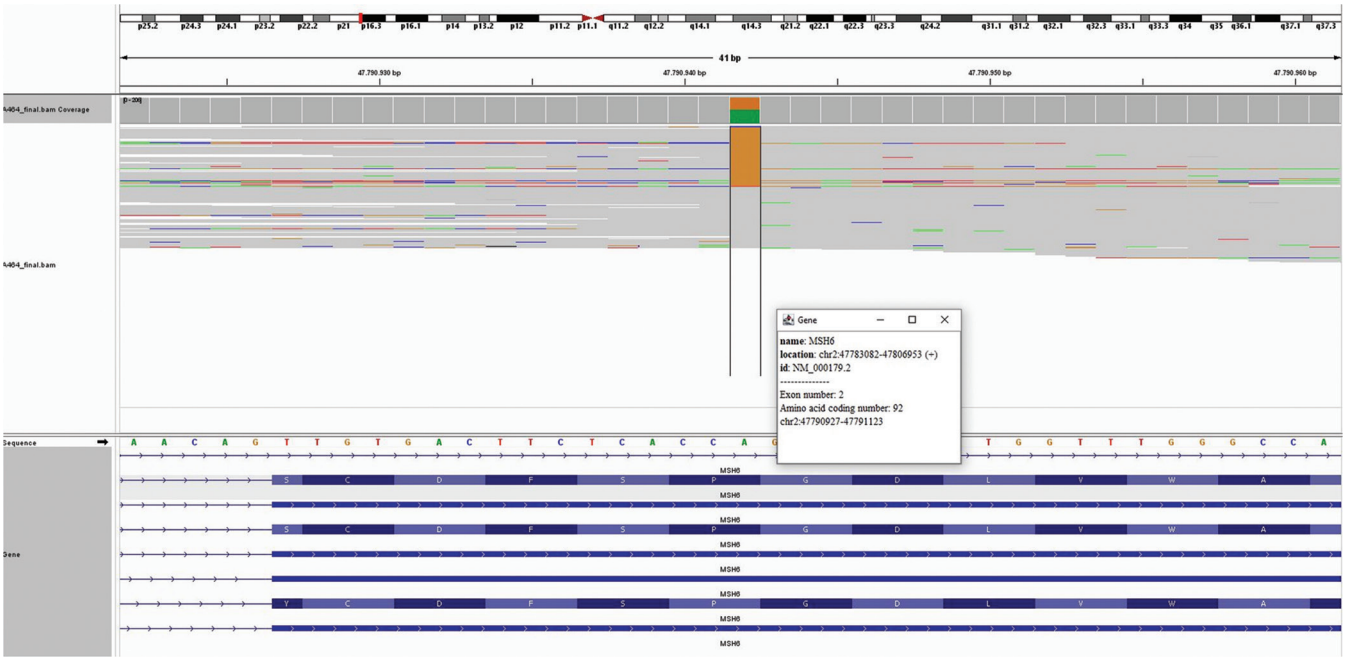

Fig. 11. Integrative Genomics Viewer image of next-generation sequencing data of MSH6 ENST00000234420.11 c.276A>G variant detected.

According to the NGS results in the present study, HOMOZYGOTE (c.215C>G) TP53 was detected as shown in Fig. 5. TP53 gene undergoes alterations considered hallmarks of tumors, and its mutation status is linked to the progression and prognosis of sporadic CRC, with a prevalence rate of 52.5% in the Arab population compared to 47.5% in a matched Western population (Youssef, et al., 2020). In addition, it has been observed that PMS2 deficiency is also linked to an increased risk of other cancers (Roberts, et al., 2018; Raza, et al., 2020).

Fig. 6 shows BRAF functions as a serine/threonine protein kinase within the mitogen-activated protein kinase (MAPK) pathway, orchestrating critical cellular processes such as proliferation, survival, differentiation, migration, and angiogenesis, and disruptions in this pathway are a fundamental driver in the development of numerous types of cancer (Caputo et al., 2019) and (Morgan et al., 2022). Based on the genomic and transcriptomic study, a BRAF mutation is found in roughly 10% of individuals diagnosed with colon cancer (Caputo, et al., 2019).

In the present study, likely pathogenic and uncertain significance mutations in the APC gene were identified as indecated in the Figs. 7-10. APC gene mutation in CRC initiates early activation of the Wnt/β-catenin pathway. Mutant APC, along with Axin2 and AMER1, disrupts the β-catenin destruction complex, leading to the accumulation

of β-catenin. This abnormal activation promotes cancer cell proliferation, invasion, and metastasis (Sanz-Pamplona, et al., 2015; Nguyen and Duong, 2018; Youssef, et al., 2020). The APC protein serves as a tumor suppressor, playing a pivotal role in both the canonical (β-catenin-dependent) Wnt signaling pathways and additional functions (Zhang, et al., 2023). Apart from its role in canonical Wnt signaling, APC contributes to the prevention and progression control of colorectal tumors (Keum and Giovannucci, 2019) and (Giannopoulou and Constantinou, 2023). It is actively participated in tasks such as chromosome segregation, the establishment of cellular polarity, migration, and the suppression of DNA replication (Stefanski and Prosperi, 2020). Most CRCs (70–80%) occur spontaneously, predominantly in individuals aged 70–75, lacking genetic predisposition or a family history. It is thought that a significant number of these sporadic cases involve somatic mutations in both alleles of the APC gene during early stages. However, about 20% of CRC patients exhibit familial aggregation, indicating a hereditary form with a family history of CRC in at least one other relative (Aghabozorgi, et al., 2019).

Fig. 11 presents the phosphatidylinositol-3-kinase (PI3K) is part of a lipid kinase family characterized by its heterodimeric structure composed of both regulatory and catalytic subunits (Cathomas, 2014). PI3K operates by phosphorylating phosphatidylinositol, a critical component of the cell membrane and a pivotal second messenger in cellular signaling pathways (He, et al., 2022). This versatile kinase plays a central role in modulating a wide array of cellular processes, encompassing proliferation, survival, apoptosis, migration, and metabolism regulation (Rakesh, et al., 2022). A prior investigation established that PIK3CA is responsible for encoding the catalytic component of phosphatidylinositol 3-kinase α (PI3Kα), which undergoes mutations in a broad spectrum of human malignancies, comprising approximately 30% of cases in CRC (Zhao, et al., 2019).

IV. Conclusions

Utilization of NGS technology in the sequencing platform has enabled to acquisition of extensive data on genetic alterations and specific gene mutations within colorectal tumor samples. This valuable information holds the potential to significantly inform clinical decision-making, aiding in the development of treatment strategies to optimize patient outcomes in the clinical setting.

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