

ORIGINAL ARTICLE

Identifying Common Mutations in Colorectal Cancer Using a 7-Gene Panel by Next Generation Sequencing

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ABSTRACT

Introduction: Colorectal cancer (CRC) arises from the cumulative effects of genetic and epigenetic alterations. Current treatment of metastatic CRC relies on combination of chemotherapy and targeted therapies such as anti-EGFR therapies. The success of targeted therapies relies on the detection of actionable targets and predictive biomarkers of resistance. The study aims to determine mutations in common actionable targets and predictive biomarkers of resistance to anti-EGFR therapies in Malaysian CRC patients. **Methods:** Mutations in 10 CRC tissues were determined by next-generation sequencing with a panel of 7 cancer-related genes covering all exons in *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, *TP53*, *NRAS*, and *EGFR* genes. Immunohistochemistry was used to determine mismatch repair (MMR) status. **Results:** Of the ten samples, 5 and 4 samples harboured two and one mutation, respectively and one had no mutation. All were missense mutations and were in five genes, namely, *KRAS*, *PIK3CA*, *TP53*, *BRAF*, and *EGFR*. They were, G12D, G12V, G12A, G13D, and V14I in *KRAS*, E545K, K733R, and D1056N in *PIK3CA*, G199V, D259Y, and R282W in *TP53*, V600E in *BRAF* and G696R in *EGFR*. Deficient mismatch repair (dMMR) was detected in three samples, of which two had *KRAS* mutation. **Conclusion:** Mutations in *KRAS* codon 12 and 13, *BRAF* and *PIK3CA* which predict resistance to anti-EGFR therapies and three *TP53* mutations were found. This is the first report of *EGFR* mutation in Malaysian CRC patients. It is predicted to be a pathogenic variant. dMMR, one of the biomarkers for treatment with immune checkpoint inhibitor was also detected.

Keywords: Colorectal carcinoma (CRC), Next-generation sequencing (NGS), mismatch repair (MMR), Microsatellite Instability (MSI), mutation

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INTRODUCTION

Colorectal cancer (CRC) is a malignancy of the colon and rectum. It is a large group of heterogeneous diseases classified under the same category where the most common groups are sporadic CRC, familial CRC, and hereditary CRC. It is the third most common cancer in the world after lung and breast cancer (1). In 2012, nearly 1.4 million new cases of CRC were diagnosed around the world (1). In Malaysia, a total of 13,693 CRC cases was reported from 2007 to 2011 making CRC the most common cancer in male and the second most common cancer in female (2).

CRC arises from the cumulative effect of genetic and epigenetic alterations. Three distinct genomic

instability pathways involved in CRC progression are chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype pathways (CIMP)(3). CIN occurs in 70%-85% CRC cases which leads to gene deletions, duplications, and chromosomal rearrangements. MSI is caused by DNA mismatch repair (MMR) deficiency resulting in mutations at simple nucleotide repeat sequences. Approximately 15% sporadic CRCs are classified as MSI (4). CIMP, on the other hand, is characterized by epigenetic instability, usually promoter hypermethylation (3).

Although many mutated genes have been reported in CRC, only fewer than 15 mutated genes are being considered as driver genes. These driver genes include *tumour suppressor protein p53 (TP53)*, *Kirsten rat sarcoma viral oncogene homolog (KRAS)*, *adenomatous polyposis coli (APC)*, *phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA)*, and *F-Box And WD Repeat Domain Containing 7 (FBXW7)* (5). The Cancer Genome Atlas Network has identified 32 recurrent

somatic mutations in CRC, with *APC*, *TP53*, *KRAS*, *PIK3CA*, *FBXW7*, *SMAD4*, *TGFBFR2* (*transforming growth factor- β receptor 2*), and *BRAF* (*proto-oncogene B-RAF*) being amongst the most frequently mutated genes (6). In Malaysian CRCs, mutations in *APC*, *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, and *TP53* have been reported in separate studies (7)(8)(9)(10).

Next-generation sequencing (NGS) technology has enormous potential in cancer research. Development of cancer-specific gene panels has increased the potential of their utility in clinical diagnostics for selecting targeted therapies such as anti-EGFR therapies by detecting multiple mutations simultaneously thus increased the time and cost effectiveness of mutation detection test (11).

In this pilot study, next-generation sequencing was performed across a 7 cancer-related gene panel using 10 CRC tissues. These 7 genes (*KRAS*, *BRAF*, *PIK3CA*, *PTEN*, *TP53*, *NRAS*, and *EGFR*) have been found to predict resistance to anti-EGFR therapies in metastatic CRC (12)(13)(14). These somatic variants are well-known and well-characterized hotspot mutations. They are also categorized as common actionable or clinically relevant somatic mutations. In clinical practice, patients with any known *KRAS* or *NRAS* mutation should not be treated with anti-EGFR therapies, while a *BRAF* inhibitor should be added in the anti-EGFR therapy for patients with *BRAF* mutation to overcome resistance. This pilot study attempted to predict the common actionable targets and those that are clinically relevant as predictive markers of resistance to anti-EGFR therapies in Malaysian CRC patients. The mutations identified will be valuable for designing new gene panels or assays for future mutation testing in Malaysia CRC population. This information will also be useful serving as negative predictive biomarker for anti-EGFR therapies in metastatic CRC patients.

MATERIALS AND METHODS

Tissue specimens and ethics statement

Formalin-fixed, paraffin-embedded (FFPE) tissues from 10 CRC patients were obtained from the Hospital of Kuala Lumpur, Malaysia. The hematoxylin and eosin (H&E)-stained tissue slides were reviewed by a pathologist to ensure sufficient tumour content. Clinicopathological and demographic data of each patient were collected from the medical records in the hospital. Ethical clearance from the National Medical Ethics Committee (NMRR-12-435-11565) was obtained for this study.

Next-generation sequencing

The FFPE tissues were sectioned at a thickness of 4 μ m using the Leica rotation microtome. Genomic DNA was extracted from a total of 20- μ m-thick tissue sections using GeneAll FFPE extraction kit according to the manufacturer's guide. The extracted DNA was quantified using the Qubit fluorometer. Libraries were

generated from 10 ng of DNA using Ion AmpliSeq library preparation kit (Thermo Fisher Scientific) and multiple primer pairs that amplify all exonic regions of *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, *TP53*, *NRAS*, and *EGFR* genes. Library quality control and quantification were done using the Agilent Bioanalyzer 2100 High Sensitivity DNA chips. The libraries were attached to the Ion Sphere particles and enriched using the Ion OneTouch 2 System with ES (Enrichment System) module (Thermo Fisher Scientific). Purified beads were loaded onto the Ion Torrent 316v2 chip. Sequencing was performed using the Ion Torrent PGM system for 200 cycles. Coverage depth of the sequencing was \sim 500x. Sequencing quality control was done on-board with Torrent Suite Software. Variants were called using Torrent Variant Caller. The variants were filtered by in-house, proprietary software, which compares the variants to those identified in COSMIC, UniProt, PolyPhen, and PubMed to produce a reduced, functional list of potentially deleterious variants and variants in oncogenes.

Immunohistochemical staining and evaluation

The 4- μ m-thick paraffin-embedded tissue sections were dewaxed and hydrated before boiling in 10 mM sodium citrate buffer (pH6.0 for MSH6) or 10 mM Tris, 1 mM ethylenediamine tetraacetic acid (EDTA) buffer (pH 9.0 for PMS2) for 20 minutes using microwave oven. The tissue sections were then incubated with 3% bovine serum albumin (BSA) for 1 hour after blocking the endogenous peroxidase with 3% hydrogen peroxide. Monoclonal primary antibodies, PMS2 (clone EP51, Dako Cytomation, Glostrup, Denmark, 1:100, 2 hours) or MSH6 (clone EP49, Dako Cytomation, Glostrup, Denmark, 1:200, 1 hour) was applied to tissue sections. The immunoreactivity detection was performed using the UltraVision Labeled Polymer (horseradish peroxidase) followed by the 3,3-diaminobenzidine (DAB) solution for colour development, according to the manufacturer's instructions (Lab Vision; Thermo Fisher Scientific, Fremont, CA, USA). Tissue sections were then counterstained with hematoxylin, dehydrated, and mounted. Negative controls were prepared by substituting the primary antibodies with Tris-buffered saline (TBS). The staining of MSH6 and PMS2 was considered positive when more than 10% of nuclear staining was observed. MMR was considered deficient when either MSH6, PMS2 or both staining in the tumour tissue was negative.

RESULTS

The demographic background characteristics of the patients are presented in Table I. The overall median age was 69 years old. Sixty percent of the patients were male and 40% were female. There were four patients in Dukes' B and three patients in Dukes' C stage. The majority of tumours were moderately differentiated (seven out of ten) and located at the right side of the colon (eight out of ten) (Table I).

Table I: Clinicopathological features of CRC patients

Sample ID	Gender	Age	Histological Grade	Stage		Tumour site
				Dukes'	TNM staging	
001	F	75	Moderately Differentiated	C	T3N2Mx	Right-sided
002	M	68	Moderately Differentiated	NA ¹	T4N1M1	Right-sided
003	M	64	Moderately Differentiated	C	T3N1M0	Right-sided
004	M	76	Moderately Differentiated	B	T3N0Mx	Left-sided
005	M	59	Moderately Differentiated	B	T3N0Mx	Right-sided
006	F	69	Moderately Differentiated	NA ¹	T3N0M0	Left-sided
007	F	71	Well Differentiated	B	T3N0Mx	Right-sided
008	M	69	NA	NA ¹	T3N0M	Right-sided
009	F	60	Moderately Differentiated	B	T4N0Mx	Right-sided
010	M	82	NA	C2	T4N2M	Right-sided

¹ NA- Not Available

A total of 2.6 million reads for 10 CRC DNA samples with 66% of loading density were obtained from the result. After eliminating loading, enrichment and clonality factors, a final of 80% of the library reads were used to perform bioinformatics analysis. Twenty percent of the reads were filtered due to test fragments, adapter dimer and low quality. All CRC DNA samples had more than 95% of total bases equal or above Phred Score 20 ($\geq Q20$) which is the default setting for data analysis with the Ion Torrent PGM instrument. Only reads with bases more than Q20 were selected to perform downstream analysis, where 99.2% of these reads were aligned to reference Human Genome 19 (Hg 19). Samples 006, 007 and 008 had significantly lower numbers of reads compared to other samples.

A total of 13 mutations were detected in five genes, namely, *KRAS*, *PIK3CA*, *TP53*, *BRAF*, and *EGFR*. All mutations detected in this study were missense mutations. Among the nine samples detected with mutations, five samples harboured two mutations while four samples harboured one mutation. Three genes, *KRAS*, *PIK3CA* and *TP53* were altered in at least three samples. The most common mutation was *KRAS* mutation, where three samples harboured mutations at codon 12, two samples at codon 13 and one sample had a mutation at codon 14. These mutations were G12D, G12V, G12A, G13D, and V14I. Three *PIK3CA* mutations were detected in this study, resulting in amino acid changes, E545K, K733R, and D1056N. In *TP53*, the detected mutations were G199V, D259Y and R282W. In this study, *BRAF* mutation (V600E) and *EGFR* mutation (G696R) were detected in only one sample each. No mutations were detected in one of the samples (Table II).

In this study, positive immunoreactivity for MSH6 protein was detected in nine samples (90%) (Figure 1)

Table II Type of mutations in Gene, Base and Amino Acid change and MSI status

Sample ID.	Gene	Type of mutation	Chromosome : Position	Base change	Amino Acid change	MSI status
001	KRAS	MISSENSE	12 : 25398284	C -> T	G12D	No
	PIK3CA	MISSENSE	3 : 178936091	G -> A	E545K	
002	BRAF	MISSENSE	7 : 140453136	A -> T	V600E	No
	TP53	MISSENSE	17 : 7577506	C -> A	D259Y	
003	KRAS	MISSENSE	12 : 25398284	C -> G	G12A	No
004	EGFR	MISSENSE	7 : 55241638	G -> A	G696R	No
	PIK3CA	MISSENSE	3 : 178952111	G -> A	D1056N	
005	KRAS	MISSENSE	12 : 25398279	C -> T	V14I	Yes
006	KRAS	MISSENSE	12 : 25398284	C -> A	G12V	No
007	PIK3CA	MISSENSE	3 : 178941879	A -> G	K733R	No
	TP53	MISSENSE	17 : 7578253	C -> A	G199V	
008	No mutation detected					Yes
009	KRAS	MISSENSE	12 : 25398281	C -> T	G13D	No
	TP53	MISSENSE	17 : 7577094	G -> A	R282W	
010	KRAS	MISSENSE	12 : 25398281	C -> T	G13D	Yes

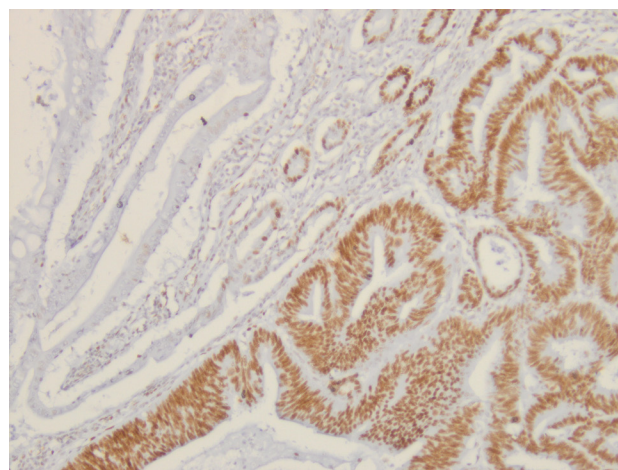


Figure 1: Positive staining of MSH6 in nuclei of tumour tissue (100x magnification)

and not detected in one samples (10%) (Figure 2). For PMS2 protein, positive immunoreactivity was detected in eight samples (80%) (Figure 3) and not detected in two samples (20%) (Figure 4). None of the samples exhibited negative immunoreactivity to both MSH6 and PMS2. Three samples with PMS2 or MSH6 negative immunoreactivity were considered MSI positive. Of these 3 samples, two had a *KRAS* mutation, whereas the remaining sample did not harbour any mutations in the seven genes tested (Table II).

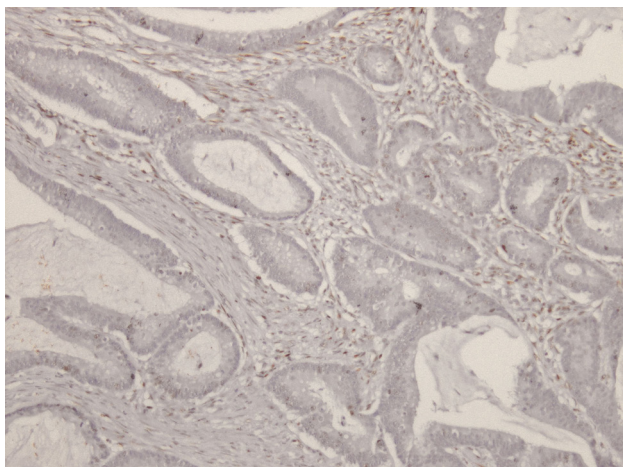


Figure 2: Absence of MSH6 staining in nuclei of tumour tissue but with positive internal control staining of lymphocytes in the stroma (100x magnification)

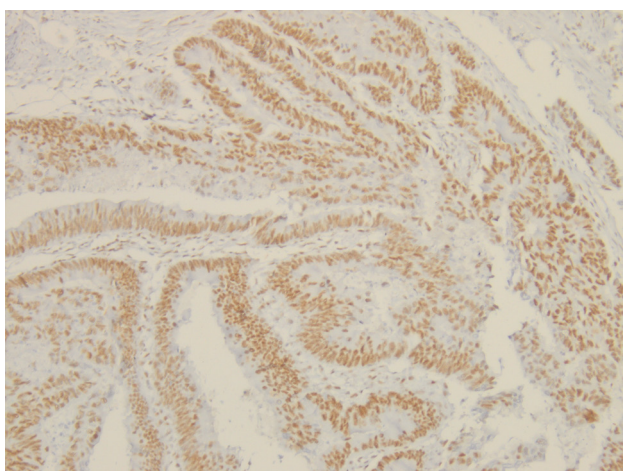


Figure 3: Positive staining of PMS2 in nuclei of tumour tissue (100x magnification)

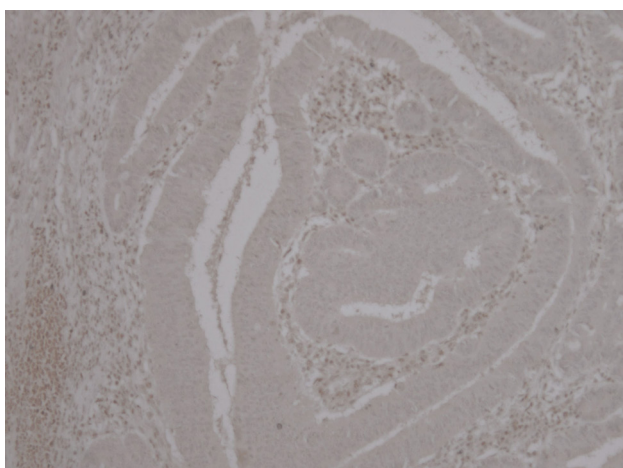


Figure 4: Absence of PMS2 staining in nuclei of tumour tissue but with positive internal control staining of lymphocytes in the stroma (100x magnification)

DISCUSSION

NGS-based gene panel test utilized NGS technology to achieve a faster and cost-effective way to investigate genetic alteration in cancer and assist in selecting relevant molecular-targeted drugs for the patient. This

technology takes a step closer to “precision medicine” where the treatment provided is tailored to the genomic information of the patient. The genomic profile of a population is the foundation of developing a gene panel. This study attempts to predict the common targetable mutations and predictive biomarker of resistance to anti-EGFR therapies in Malaysian CRC patients.

In this study, mutations in seven genes in CRC was screened using NGS with a 7-cancer gene panel. These 7 genes are commonly altered in CRC population and have been reported to predict resistance to anti-EGFR therapies in metastatic CRC patients. Overall, 90% (9/10) of the samples harboured at least one mutation.

The most frequent mutation identified in this study is the *KRAS* gene. Consistent with the previous studies in Malaysia (8), four hotspot mutations of *KRAS* (G12A, G12D, G12V, and G13D) was found in 6 CRC samples. These mutations localized in the GTP nucleotide binding domain impair GTPase activity rendering mutant *KRAS* protein persistently active, thereby activating the downstream signaling pathways. Karapetis et al. and Amado et al., separately reported that CRC patients with these *KRAS* mutations do not benefit from cetuximab and panitumumab (15)(16). Our study also found a mutant variant, *KRAS* V14I in the GTP-binding region. This mutation has a low frequency in CRC but has been reported to reduce *KRAS* GTPase activity leading to its constitutive activation and upregulation of downstream ERK signaling (17).

The majority of *KRAS* mutations occur at codons 12, 13, and 61 resulting in constitutive activation of *KRAS* protein (18), which promotes proliferation and inhibits apoptosis of cancer cells (19). *KRAS* mutation was found in 35%-45% of CRCs (20). The mutation status of *KRAS* is a predictive biomarker for anti-EGFR therapy in CRC. Metastatic CRCs (mCRC) with mutant *KRAS* are resistant to cetuximab and panitumumab (approved anti-EGFR drugs) (21)(22). In the first-line treatment of mCRC, the addition of cetuximab to FOLFOX [folinic acid (leucovorin), fluorouracil (5-FU), oxaliplatin]/FOLFIRI [folinic acid (leucovorin), fluorouracil (5-FU), irinotecan (Camptosar)] does not improve the progression-free survival (PFS) or overall survival (OS) of the patients with mutant *KRAS* (23).

PIK3CA gene is frequently mutated in human cancers with a frequency of 32% in CRC (24). The *PIK3CA* hotspot mutations are localized in exons 9 and 20. Tumours with *PIK3CA* mutant variants were found to be resistant to cetuximab and panitumumab (25). However, the predictive role of *PIK3CA* mutations in anti-EGFR therapies remains controversial because *PIK3CA* mutations are usually found concomitantly with *KRAS* mutations (26). In this study, three *PIK3CA* mutant variants were detected. The mutant variant E545K in exon 9, which encodes the helical domain of PI3K

catalytic subunit α protein, is one of the most common and strong oncogenic mutations in CRC. This mutant variant has the ability to activate PI3K signaling and MEK1/2 module of mitogen-activated protein kinase (MAPK) pathway (27). *PIK3CA* D1056N mutation is localized in exon 20, which encodes the kinase domain of PI3K catalytic subunit α protein. Lee et al. have shown that a patient with *KRAS* (G12D) mutant mCRC that also harboured *PIK3CA* D1056N mutation had stable disease for a longer period compared to other *PIK3CA* mutant variants when receiving simvastatin/cetuximab/irinotecan treatment (28). A systematic review and meta-analysis also suggested that a mutation in *PIK3CA* exon 20 is a potentially better predictor biomarker for resistance to anti-EGFR therapy in *KRAS* wild-type mCRC (29). *PIK3CA* K733R mutation in exon 14 which was previously discovered in CRC (30) was also detected in this study. This mutation has been predicted to be a pathogenic mutation in the Cosmic Sanger database.

TP53 (tumour suppressor p53) or p53 is the most frequent mutated gene in the TCGA Pan-Cancer cohort (31). In CRC, the frequency of *TP53* mutations ranges from 30%~45%. Mutations of *TP53* are mainly observed at the DNA binding domain in exon 5 to exon 8 (32). The mutation of *TP53* not only results in loss of tumour-suppressive properties but also gain of new oncogenic activity. These activities include the promotion of cell growth, migration, invasion and metastasis, and chemoresistance (33). Sui and coworkers have shown that JNK activation promotes autophagy most probably via inducing Bcl-2 phosphorylation causing colon cancer cells to be resistant to 5-FU *in-vitro* (34). In this study, three *TP53* mutations were detected, namely G199V (33.3%), D259Y (33.3%) and R282W (33.3%). The p.R282W mutant variant is a hotspot mutation that is localized within the DNA binding domain of the p53 protein. This mutant variant inhibits AMPK (adenosine monophosphate (AMP)-activated protein kinase) activation, promotes invasive cell growth during tumour development, inhibits activity of p63 and p73, reduces metastasis suppressor function of Kruppel-like-factor 17 (KLF17), promotes cellular transformation and invasion, and has chemoresistant properties (35)(36). The p.G199V is a mutant variant that has antiapoptotic function via the signal transducer and activator of transcription-3 (STAT3) pathway in KAT-18 cell line (37). The p.D259Y mutant variant is also localized within the DNA binding domain of p53 protein and has been reported in patients with Crohn's disease (38).

The *BRAF* gene is an important player in MAPK/ERKs signaling pathway affecting cell division, differentiation, and secretion. In CRC, the frequency of *BRAF* mutation ranged from 5%-11% (39). A systemic review revealed that patients with *BRAF* mutations have a poorer overall survival (OS) compared to wild-type *BRAF* (40). In a clinical study, patients with *BRAF* mutations receiving

either FOLFIRI plus cetuximab or FOLFIRI has been associated with shorter OS (41). In this study, the most prevalent mutation, V600E, was detected in one sample. This mutant variant, localized within the activation segment of the kinase domain of *BRAF* protein, is an activation mutation which accounts for around 90% of all *BRAF* mutations in CRC. The V600E is also a predictive biomarker in the treatment of CRC patients as this mutant variant is resistant to *BRAF* inhibitors in cell line studies (42)(43).

Epidermal growth factor receptor (EGFR) gene, is a low prevalence gene mutation in CRC but in certain populations, the mutation frequencies can range from 11~22% (44)(45). Although overall low in prevalence, chemoresistance to cetuximab was discovered in certain *EGFR* rare variants (46)(47). In this study, one *EGFR* mutation (p.G696R) was detected. To the best of our knowledge, this is the first time *EGFR* mutation is reported in a Malaysian CRC study. The p.G696R discovered is localized within the cytoplasmic domain of *EGFR* protein and was previously discovered in non-small cell lung cancer (48). This mutant variant has not been previously reported in CRC during the time of analysis. To the best of our knowledge, there is limited information on this variant. However, using different mutation predicting software (MutPred score=0.805, Provean score=-6.569, PMUT score=0.61 and PolyPen-2 score=0.999), the score of these tests suggested this mutant variant to be pathogenic.

In this study, MSH6 and PMS2 antibodies were used to detect the status of MMR in tumour tissue. MMR was considered deficient when there was a loss of MSH6 and/or PMS2 protein expression in the tumour tissue. This method was proposed by Shia et al., where the predictive value of IHC, using PMS2 and MSH6 antibodies, is nearly equivalent to MSI testing (49).

MSI/MMR has been found to be a predictive biomarker of treatment response to immune checkpoint inhibitors for colorectal carcinoma. Colorectal carcinoma patients with high MSI or deficient MMR was reported to response better to anti PD-L1 immunotherapy (pembrolizumab) compared to patients with low/no MSI or proficient MMR (12). MSI was also reported to have association with *BRAF*, *TP53*, *PTEN*, and *KRAS* mutations. Study by Albitar et al. proposed that the effectiveness of therapy targeting MSI and *BRAF* and *PTEN* mutation may increase if combined with checkpoint inhibitors (50).

There were three samples (006, 007 and 008) with a lower number of reads compared to other samples in this study. An insufficient number of reads may affect the detection of rare mutation with low allelic frequency. This was probably due to inaccurate quantification that affects the molarity of samples during pooling or the nature of the samples (FFPE tissues) that affected the

sequencing quality.

The limitations of this study are that the sample size was small and the mutations detected by NGS were not validated by other tests. It is worthwhile to further examine and validate the *EGFR* mutant variant in a larger sample size with matched tumour and normal tissues to define whether it is a somatic or germline mutation. The types/sites of variant that we found in *KRAS*, *PIK3CA*, *TP53*, and *BRAF* often represent somatic variants. These somatic variants are well-known and well-characterized hotspot mutations.

CONCLUSION

We have identified mutations in *KRAS* codon 12 and 13, *BRAF*, and *PIK3CA* but not *NRAS* and *PTEN*. These genes can predict resistance to anti-EGFR therapies in CRC. Three *TP53* mutations were found. This is the first report of EGFR mutation (G696R) in Malaysia CRC population which is predicted to be a pathogenic variant. More samples need to be tested to further support these findings. This study has provided information on the potential clinical utility of this 7-cancer gene panel and is also useful for designing new gene panels with an increased number of genes for future mutation testing.

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