KRAS GENE MUTATIONAL PROFILES AMONG COLORECTAL CANCER (CRC) PATIENTS IN HOSPITAL UNIVERSITI SAINS MALAYSIA (USM)

DR. HIDAYATI HUSAINY BINTI HASBULLAH

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

AJCC	American Joint Committee on Cancer
CEA	Carcinoembryonic antigen
CIN	Chromosomal instability
CNV	Copy number variants
CRC	Colorectal cancer
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EGFR	Epithelial growth factor receptor
FFPE	Fresh-frozen-paraffin-embedded
mCRC	Metastatic colorectal cancer
MSI	Microsatellite instability
OS	Overall survival
PCR	Polymerase chain reaction
PFS	Progression-free survival
Taq	*Thermophilus aquaticus*
TBE	Tris-borate- EDTA

SYMBOLS

bp	basepairs
df	degree of freedom
mL	millilitre
mM	millimolar
µl	microlitre
µM	micromolar
rpm	revolutions per minute
U	units
°C	degree Celsius
%	percent
≤	less or equal than
>	more than

ABSTRAK

PROFIL MUTASI GEN *KRAS* DI KALANGAN PESAKIT KANSER KOLON DAN REKTUM DI HOSPITAL UNIVERSITI SAINS MALAYSIA (USM)

Pengenalan

Beban penyakit kanser kolon dan rektum di kalangan penghidap penyakit ini menyebakan terdapat keperluan yang segera untuk menghasilkan suatu penanda biologi yang mampu menambah baik pengesanan, rawatan dan/atau kelangsungan hidup pesakit kanser ini. Salah satu penanda biologi tersebut adalah gen *KRAS*. Kajian telah membuktikan bahawa status mutasi *KRAS* adalah penanda yang cukup berkesan untuk menentukan respon kepada ubat monoklonal antibodi anti-EGFR. Terkini, FDA telah meluluskan penggunaan sotorasib, sejenis ubat perencat *KRAS*-G12C protein untuk kegunaan pesakit kanser paru-paru yang mempunyai mutasi G12C. Walaupun pelbagai kajian tentang gen *KRAS* telah dijalankan di peringkat antarabangsa, data tentang profil mutasi *KRAS* di kalangan pesakit di Malaysia masih terhad. Kajian ini akan cuba untuk mendalami profil mutasi gen *KRAS* di kalangan pesakit kanser kolon dan rektum di Hospital USM, Kelantan yang terletak di pantai timur Malaysia.

Metodologi

Kajian rentas-silang ini melibatkan pesakit-pesakit kanser kolon dan rektum yang menerima rawatan di Hospital USM antara tahun 2018 hingga 2019. DNA genomik telah diekstrak dari 33 blok tisu kanser kolon dan rektum yang telah diawet formalin dan terbenam dalam lilin paraffin. Kodon 12 dan 13 gen *KRAS* telah diamplifikasi

menggunakan kaedah tindak balas berantai polimerase (PCR). Hasil PCR produk ini dihantar ke makmal komersial untuk penjujukan DNA Sanger.

Keputusan

Kajian ini mengenalpasti bahawa kadar mutasi gen *KRAS* di kodon 12 dan 13 adalah sebanyak 36.4%. G12D (50.0%, 6/12) adalah mutasi yang paling kerap ditemui, diikuti G12V (25.0%, 3/12); G13D (16.7%, 2/12) dan G12S (8.3%, 1/12). Tidak ada perbezaan yang signifikan antara jantina, umur dan etnik di antara pesakit yang mempunyai mutasi dan yang tidak mempunyai mutasi di gen *KRAS* ($p = 0.469$; $p = 0.392$; $p = 0.630$). Kajian ini juga mendapati bahawa tidak ada kolerasi antara mutasi *KRAS* dan ciri-ciri kanser itu sendiri seperti pembahagian histologi dan tahap kanser, lokasi kanser utama, dan bacaan awal CEA ($p = 0.420$; $p = >0.950$; $p = 0.429$).

Kesimpulan

Kadar mutasi gen *KRAS* di kalangan pesakit kanser kolon dan rektal di Hospital USM adalah signifikan. Kajian ini adalah kajian pertama yang membuktikan bahawa kekerapan mutasi *KRAS* adalah lebih tinggi di pantai timur Malaysia berbanding kajian lepas yang dijalankan di pantai barat Malaysia. Variasi mutasi *KRAS* di kalangan pesakit kami adalah konsisten dengan kajian-kajian yang lepas. Kajian ini juga mendapati bahawa tiada perkaitan antara mutasi gen *KRAS* dan ciri-ciri patologi dan klinikal pesakit kanser kolon dan rektum.

Kata Kunci: Kanser kolon dan rektum, *KRAS*, Kodon 12 dan 13

ABSTRACT

KRAS GENE MUTATIONAL PROFILES AMONG COLORECTAL CANCER (CRC) PATIENTS IN HOSPITAL UNIVERSITI SAINS MALAYSIA (USM)

Introduction

The global burden of colorectal cancer (CRC) necessitates the development of a novel biomarker that aims to improve the detection, management and/or treatment outcomes of CRC patients. One such marker is the *KRAS* oncogene. Studies have shown that *KRAS* mutational status is a robust predictive marker for response to anti-EGFR monoclonal antibodies. Recently, FDA has approved the use of sotorasib, a *KRAS*-G12C protein inhibitor in non-small cell lung carcinoma patients with G12C mutations. The role of sotorasib in CRC is still under evaluation but has shown great potential. Despite extensive research worldwide, there is still limited data on the *KRAS* mutational profiles amongst CRC patients in Malaysia. This study aims to determine the proportion of CRC patients with *KRAS* mutations at codons 12 and 13 in HUSM, Kelantan located on the East Coast of Malaysia, and to ascertain if there is any correlation between known *KRAS* mutations and clinicopathological features of CRC.

Methodology

This is a cross-sectional, non-interventional genetic study involving CRC patients diagnosed in Hospital USM, Kelantan between 2018 and 2019. DNA was extracted from formalin-fixed, paraffin-embedded tissues of the primary CRC tumour (n=33) after informed consent was obtained. Codons 12 and 13 of the *KRAS* gene were amplified using

the conventional polymerase chain reaction (PCR) method. The PCR products were outsourced to a commercial laboratory for DNA Sanger sequencing.

Result

Current study identified that the frequency of *KRAS* mutations at codons 12 and 13 was 36.4% (12/33). G12D (50.0%, 6/12) was the most common single point mutation observed followed by G12V (25.0%, 3/12); G13D (16.7%, 2/12) and G12S (8.3%, 1/12). No significant differences were observed in the distribution of gender, age and ethnicity between patients with mutant *KRAS* and wild-type *KRAS* ($p = 0.469$; $p = 0.392$; $p = 0.630$, respectively). There was also no correlation between *KRAS* mutations and tumour characteristics such as the stage and histological differentiation, location of the primary tumour and initial CEA level ($p = 0.420$; $p = >0.950$; $p = 0.429$, respectively).

Conclusion

A significant proportion of CRC patients in Hospital USM had *KRAS* mutations. This study is the first to demonstrate that the frequency of *KRAS* mutations is higher in our region compared to the previous studies conducted at the West Coast of Malaysia. The spectrum of *KRAS* mutations in the study cohort is consistent with previous reports. No association between *KRAS* mutations and any of the clinicopathological features of CRC patients was observed.

Keywords: Colorectal cancer, *KRAS*, Codons 12 and 13

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide and it is one of the leading causes of cancer death, second only to lung cancer [1]. By 2030, CRC cases are predicted to increase by 60% to more than 2.2 million new cases and 1.1 million deaths [2]. In Malaysia, CRC is the most common cancer diagnosed in males and second in females according to Malaysia National Cancer Registry 2012-2016 [3]. The majority of CRC cases are diagnosed at the advanced stage (III or IV) of the malignancy. This contributes to the high morbidity and mortality due to this disease.

The global burden of CRC necessitates the development of a novel biomarker that aims to improve the detection, management and/or treatment outcome of patients diagnosed with this cancer. One such marker is *KRAS* proto-oncogene, GTPase. Recent studies have shown that *KRAS* mutational status is a robust predictive marker for response to anti-EGFR monoclonal antibodies, namely cetuximab and panitumumab. These EGFR-directed therapies are now being used routinely as first-line and subsequent-line therapy for metastatic CRC in combination with standard chemotherapy. Patients with advanced CRC who exhibited mutant *KRAS* in the tumour tissues are resistant to the targeted anti-EGFR therapy [4,5]. Currently, research is ongoing to identify therapeutic strategies that specifically target the *KRAS* activation pathway and successfully inhibit its activation.

KRAS or its previous name, Kirsten rat sarcoma viral oncogene homolog, is a member of the RAS type GTPase family of genes. The gene encodes KRAS protein – a small GTPase transductor protein, located downstream from EGFR in the KRAS-BRAF-MEK-ERK

pathway. This pathway is one of the most important mitogen-activated protein kinase (MAPK) signalling pathways involved in cell proliferation and differentiation [6]. Mutations in *KRAS* result in EGFR-independent activation of the MAPK pathway by reducing the GTPase activity. This will lead to an increase in uncontrolled cell proliferation, potentially occurring in the early stages of colorectal carcinogenesis as postulated in the chromosomal instability (CIN) pathway [7]. CIN is the most important pathway for the development of CRC, representing up to 80% of sporadic CRC cases. Mutation in *KRAS* is proposed to drive the progression of a small adenoma to a large adenoma before further genetic alterations result in the development of carcinoma and its progression.

To date, more than 90% of the activating *KRAS* mutations identified in CRC occurred at codons 12 and 13 in exon 2 of the gene. Single base substitution of glycine to aspartate (G12D) and glycine to valine (G12V) on codon 12 are the most common mutations observed [8,9]. Although mutations at exons 3 and 4 have also been recorded, these accounted for only about 1-4% of *KRAS* mutations [9–11].

KRAS mutational rates among CRC patients vary according to the population studied, ranging from 30 to 52% [5,12,13]. The explanation for the differences in the prevalence of *KRAS* mutations among CRC patients across the globe is still unknown and no definite pattern and predisposing factors have been observed. In Malaysia, the prevalence is lower (approximately 20%) as previously reported [14–16]. Despite this, *KRAS* remained as one of the most common genes mutated in CRC patients in Malaysia [17]. These previous studies however were limited to CRC patients who underwent surgical treatment in Kuala

Lumpur and Selangor. The population demographic of these two states on the West Coast of Malaysia is strikingly different from those in Kelantan. Kuala Lumpur for instance has a population comprised of approximately 40% Malays, 40% Chinese and 10% Indians. In contrast, more than 90% of Kelantanese are Malays while Chinese and Indians encompass less than 5% of the population respectively [18]. Murad et al., [14] reported that *KRAS* mutations were found more commonly in Chinese patients than ethnicities which may be due to differences in the genetic susceptibility towards CRC.

CRC cases continue to rise in Kelantan, with an increment of 16% of new cases reported from 2012-2016 in comparison to previous data from 2007-2011 [3]. In Hospital USM, recent data from the Endoscopy Unit and Pathology Department showed that around 6% of patients who underwent colonoscopy from January to August 2020 were subsequently diagnosed with CRC or colonic adenoma (unpublished data).

To the best of our knowledge, no published local study has yet that explored the *KRAS* mutational status among CRC patients in Kelantan. Knowing the mutational status of this important gene in CRC among Kelantanese patients is central to developing background data on the pattern of gene mutations involved in CRC patients in this region. This information will serve as preliminary data for further research that explores the candidate genes in the development of CRC in a bigger sample and unique population of Kelantan. In this era where precision medicine and targeted therapy is the current treatment goal for cancer, the findings of this study may also provide clinicians and pathologists with the necessary input in the development of relevant genetic testing as part of a diagnostic workup for CRC patients in Kelantan in the near future.

1.2 OBJECTIVES OF THE STUDY

1.2.1 General Objective

1. To explore the *KRAS* mutational status amongst colorectal cancer (CRC) patients in Hospital USM, Kelantan.

1.2.2. Specific Objective

1. To determine the proportion of CRC patients in Hospital USM, Kelantan with *KRAS* mutations at codon 12 or 13.
2. To determine the association between known *KRAS* mutational status at codon 12 or 13 and clinicopathological features of CRC patients in Hospital USM, Kelantan.

CHAPTER 2: STUDY PROTOCOL

2.1 STUDY PROTOCOL

Research Title: *KRAS* Gene Mutational Profiles among Colorectal Cancer (CRC) Patients in Hospital Universiti Sains Malaysia

Principal Investigator (MMC No. if applicable): Dr. Hidayati Husainy Hasbullah (MMC No: 60164)

Co-researchers (MMC No. if applicable):

1) Dr. Marahaini Musa
2) Associate Professor Dr. Sarina Sulong
3) Dr. Nur Asyilla Che Jalil (MMC No: 46153)

Introduction

Colorectal cancer (CRC) is the most common cancer diagnosed in males and second in females according to Malaysia National Cancer Registry 2012-2016. In 2016, a total of 3471 CRC cases were recorded in Malaysia of which 56.1% were among males and 43.9% were among females (National Cancer Institute, 2019). In the majority of cases, the patients presented late at stage III or IV of the malignancy. Globally, CRC is the third most common cancer and the fourth most common cancer-related death (Mármol et al., 2017). By 2030, CRC cases are predicted to increase by 60% to more than 2.2 million new cases and 1.1 million deaths (Arnold et al., 2017).

The global burden of CRC necessitates the development of novel biomarkers that aim to improve the detection, management and outcome of patients diagnosed with CRC. As more insights are obtained regarding molecular changes in colorectal carcinogenesis, there is growing interest in the establishment of a molecular marker as a novel prognostic and predictive factor in management of CRC. One such marker is *KRAS* oncogene; in which its mutation occurs early in the development of CRC (Fang and Richardson, 2005).

Up to this date, *KRAS* mutational status is an established and a robust predictive marker for response to anti-EGFR monoclonal antibodies, namely cetuximab and panitumumab. Various studies have consistently demonstrated that metastatic CRC patients with wild-type *KRAS* gene responded to the targeted anti-EGFR treatment while those with mutant *KRAS* were resistant towards the same mode of treatment (Ciardiello et al., 2009; MisaleRona, 2014; Sullivan and Kozuch, 2011). Due to its strong predictive marker, testing for *KRAS* mutation is required and mandatory before starting anti-EGFR therapy in CRC patients.

Problem statement and study rationale

CRC is a major health concern worldwide including Malaysia. The discovery of *KRAS* mutations that indicate poor response to anti-EGFR monoclonal antibody therapy paved the way for more studies on *KRAS* gene mutations to uncover other clinical relevance that may improve the detection, management and outcome of CRC patients. This includes its potential as biomarker for early diagnosis, susceptibility assessment as well as a potential target for directed and personalized therapy.

Despite being widely investigated, there is still limited data regarding *KRAS* mutational profile amongst CRC patients in Malaysia, specifically within Kelantanese population. Previous studies that were conducted in our country primarily involved CRC patients from West Coast of Malaysia, namely Selangor and Kuala Lumpur (Murad et al., 2012; Yip et al., 2013; Zulhabri et al., 2012). Apart from the prevalence and types of *KRAS* mutations, clinicopathological characteristics of CRC patients with these mutations had also been studied but their associations remain unclear. Therefore, this study aims to determine the *KRAS* mutational profiles among CRC patients in Kelantan and to discover if any, the associations between the mutations and the clinicopathological features of the tumour in these patients. This information will aid in our understanding of the genetic profile of CRC patients and may serve as a preliminary study for further research exploring the candidate genes in development of CRC in a bigger sample population in Kelantan.

Research question(s)

What is the proportion of colorectal patients in HUSM with *KRAS* mutations and are they any associations between the mutations and their clinicopathological features?

Objective

General: To explore the *KRAS* mutational status amongst colorectal cancer patients (CRC) in Hospital Universiti Sains Malaysia (Hospital USM).

Specific:

1. To determine the proportion of *KRAS* mutations in codon 12 or 13 in CRC patients in HUSM
2. To determine the association between known *KRAS* mutational status in codon 12 or 13 and clinicopathological features of CRC patients in HUSM

Literature review

Colorectal cancer is a disease that arise from multistep accumulations of genetic and epigenetic changes. Three major molecular pathways had been identified in CRC carcinogenesis – chromosome instability (CIN); microsatellite instability (MSI) and CpG

island Methylation Pathway (CIMP) (Hardiman, 2018). In up to 80% cases of sporadic CRC, the tumour arises from CIN pathway and progresses through the classic adenoma – carcinoma sequence. This pathway is triggered by multistep mutational events involving multiple genes for example, *KRAS* oncogene and tumour suppressor genes such as *APC* and *p53*. The Cancer Genome Atlas Network had identified the most frequently mutated genes in CIN pathway which include *APC, TP53, KRAS, PIK3CA, FBXW7, SMAD4, TCF7L2 and NRAS* (Muzny et al., 2012). Among these genes, *KRAS* mutations testing is the most useful assessment to-date, being used routinely to predict response to anti-EGFR therapy.

KRAS (Kirsten ras sarcoma viral oncogene) is a guanosine triphosphate (GTP)-binding protein located downstream from EGFR in the KRAS-BRAF-MEK-ERK pathway, one of the most important mitogen-activated protein kinase (MAPK) signalling pathways (Sullivan and Kozuch, 2011). **Figure 1** illustrates this pathway clearly. ERK-MAPK pathway is important in cell proliferation and differentiation. Mutations in *KRAS* results in EGFR-independent activation of MAPK pathway by reducing the GTPase activity that leads to an increase of cell proliferation potentially occurred in the early stage of colorectal carcinogenesis (Fang and Richardson, 2005). It is also proposed that ERK-MAPK pathway is involved in the progression and oncogenic behaviour of human CRC.

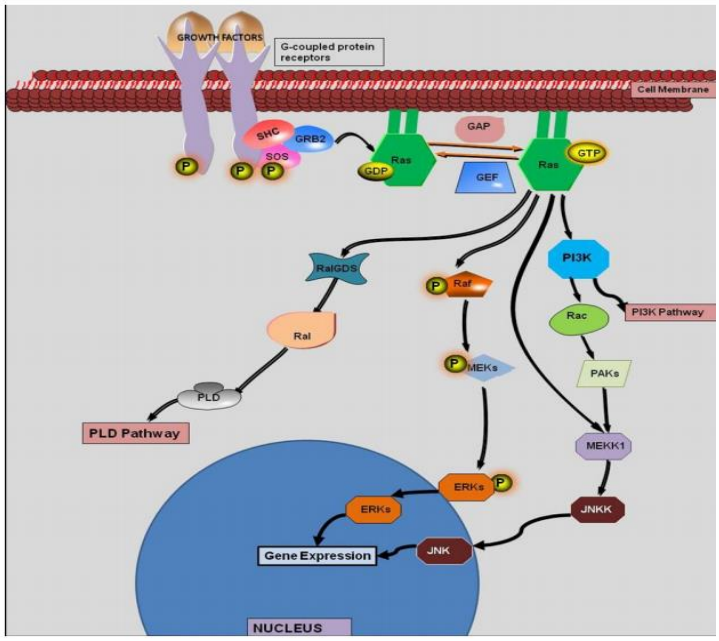


Figure 1: The Ras activation cascade (Arrington et al., 2012).

More than 90% of the activating mutations occurred at codon 12 and codon 13 in exon 2 of *KRAS* gene with single base substitution glycine to aspartate (G12D) and glycine to valine (G12V) on codon 12 being the most common mutations observed (Hayama et al., 2019; Neumann et al., 2009; Sullivan and Kozuch, 2011). Mutations at codons 61 and 146 have also been recorded although this accounts for only about 1-4% of *KRAS* mutations and its clinical significance remain unclear (Neumann et al., 2009).

Various studies have demonstrated that mutations in *KRAS* gene are implicated in approximately 30-50% of CRC patients, depending on the population studied (H. J.N. Andreyev et al., 2001; Hayama et al., 2019; Scott et al., 2020). The explanation for the differences in prevalence of *KRAS* mutations among CRC patients across the globe is still unknown and no definite pattern and predisposing factors have been observed. In Malaysia, the prevalence is lower (approximately 20%) as previously reported (Murad et al., 2012; Yip et al., 2013; Zulhabri et al., 2012). Despite this, *KRAS* remained as the most common gene mutated in CRC patients in Malaysia as demonstrated by Lee et al., (2019) using the 7-gene panel by Next Generation Sequencing (NGS). The previous studies in Malaysia however were limited to CRC patients who underwent surgical removal of the tumour in Kuala Lumpur and Selangor. The population makeup of these two states in the West Coast of Malaysia is strikingly different than those in Kelantan. Kuala Lumpur for instance, has a population comprises of approximately 40% Malays, 40% Chinese and 10% Indians. In contrast, more than 90% of Kelantanese are Malays while Chinese and Indians encompass about less than 5% of the population respectively (Malaysia, 2018). Murad et al., (2012) reported that *KRAS* mutations were found more commonly in Chinese patients compared to other ethnicities and this may be due to differences in the genetic susceptibility towards CRC. CRC cases continue to rise in Kelantan, with an increment of 16% new cases reported for the period of 2012-2016 in comparison to previous data from 2007-2011 (National Cancer Institute, 2019). In Hospital USM, a recent data from Endoscopy Unit and Pathology Department shows that around 6% of patients who underwent colonoscopy from January 2020 till August 2020 were subsequently diagnosed with CRC or adenoma, which was significantly higher than the previous year (unpublished data).

A study conducted in Hospital USM also determined that there was a high proportion of metabolic diseases (hypertension and Type 2 Diabetes Mellitus) among CRC patients in Kelantan population (Othman and Mat Zin, 2008). Interestingly, the same study also reported that patients with these metabolic diseases were more likely to present with late-stage CRC and with cancers located at the left side of the colon. Associations between *KRAS* mutational status and clinicopathological features of CRC had been studied but there is no consensus obtained regarding its relation. Hayama et al. (2019) and Scott et al. (2020) for instance demonstrated that *KRAS*-mutated CRC were more frequently observed at the right side of the colon while Zulhabri et al. (2012) reported that these mutations are more common on left-sided CRC. Other studies including the collaborative RASCAL study (H. Jervoise N. Andreyev et al., 1998) and Phipps et al. (2013) showed no association between *KRAS* mutations and tumour site.

To the best of our knowledge, there has been no published localised study yet that explores the *KRAS* mutational status among CRC patients in Kelantan and its association with clinicopathological features of these patients. Knowing the mutational status of this important gene in CRC among Kelantanese patients is central in developing background data on the pattern of gene mutations involved in CRC in our population. This information may serve as a preliminary data for further research that explores the candidate genes in development of CRC in a bigger sample and unique population of Kelantan.

Research Design

This is a 2-year Cross sectional, non-interventional genetic study

Study Population

Colorectal cancer patients who were treated with surgical resection at Hospital USM from August 2018 till December 2020.

Subject criteria

Inclusion criteria:

a) CRC patients, stage I to IV according to 8th edition of American Joint Committee on Cancer (AJCC) staging system
b) Subjects age 18 years old and above

Exclusion criteria:

a) Histology showing other than adenocarcinoma of the colon or/and rectum, for example gastrointestinal stromal tumour (GIST), carcinoid tumour, neuroendocrine tumour or lymphoma
b) History of hereditary colorectal carcinoma (i.e., familial adenomatous polyposis and non-polyposis colorectal cancer)
c) Multiple primary malignancies
d) Patient who receives neoadjuvant chemotherapy

Sample size estimation

Objective 1: Sample size using single proportion calculator estimation

Subjects: Patient underwent surgical resection for colorectal cancer at HUSM

From this (Yip et al., 2013):

Table 2. Mutation frequencies

	Mutation, nucleotide (amino acid)	Case (s)	Case no.
KRAS, n = 44	c.34G>C (G12R)	1	C43
	c.35G>A (G12D)	5	C1, C2, C5, C11, C47
	c.35G>T (G12V)	1	C8
	c.35G>C (G12A)	1	C35
	c.38G>A (G13D)	2	C39, C46
	c.183A>C (Q61H)	1	C16
BRAF, n = 43	c.1799T>A (V600E)	1	C31
PTEN, n = 43	Not found	0	-

n, number of samples that were successfully tested/evaluated.

We assumed that the prevalence of *KRAS* mutations in codon 12/13 is 22% (10/44)

Precision = 10%, Confidence level = 95%

Sample Size Calculator (web)

1 proportion - Estimation

Proportion (p):	0.22	
Precision (± proportion):	0.1	
Confidence level 100(1 - α):	95	%
Expected dropout rate:	5	%
Calculate / Reset		
Sample size, n =	66	
Sample size (with 5% dropout), n_{drop} =	70	

Formula reference:

Arifin, W. N. (2013). Introduction to sample size calculation. Education in Medicine Journal, 5(2), e89-e96.

Naing, N. N. (2003). Determination of Sample Size. The Malaysian Journal of Medical Sciences: MJMS, 10(2), 84-86.

Based on calculation, a minimum of 66 samples is required.

Objective 2: Sample size using two-proportion comparison for independent variables

Subjects: Patient underwent surgical resection for colorectal cancer at HUSM

From this (Hayama et al., 2019):

Clinicopathological feature	$n = 126$ (%)	Codon 12 $n = 57$ (%)	Codon 13 $n = 17$ (%)	P value
Age (median, range)	66, 28–90	69, 34–93	70, 50–83	0.08
Gender				
Male	84 (66.7)	29 (50.9)	11 (64.7)	0.07
Female	42 (33.3)	28 (49.1)	6 (35.3)	
T stage				
T1 or T2	42 (66.7)	13 (22.8)	3 (17.6)	0.07
T3 or T4	84 (33.3)	44 (77.2)	14 (82.4)	
N stage				
N0	79 (62.7)	33 (57.9)	9 (52.9)	0.40
N1 or N2	47 (37.3)	24 (42.1)	8 (47.1)	
Histology				
Well or moderate	116 (7.25)	53 (93.0)	16 (94.1)	0.76
Others	10 (92.8)	4 (7.0)	1 (5.9)	
Tumor location				
Right side	34 (27)	26 (45.6)	6 (35.3)	0.02
Left side	92 (73)	31 (54.4)	11 (64.7)	

We assumed that P_0 = CRC patients without *KRAS* mutations having right sided colon is 25% (34/126)

P_1 = CRC patients with *KRAS* mutations having right sided colon is 45% (32/74)

Significance level = 0.05 two-tailed, Power = 80%

Sample Size Calculator (web)

2 proportions - Hypothesis Testing

Proportion in control (p_0):	0.25	
Proportion in case (p_1):	0.45	
Significance level (α):	0.05	Two-tailed
Power ($1 - \beta$):	80	%
Expected dropout rate:	5	%
Calculate / Reset		
Sample size, n =	89	
Sample size (with 5% dropout), n_{drop} =	94	

Formula reference:

Lemeshow, S., Hosmer Jr, D. W., Klar, J., Lwanga, S. K. (1990). Adequacy of sample size in health studies. England: John Wiley & Sons Ltd.

Based on this calculation, a sample size of 89 is required.

Combining the calculations for sample size of the 2 specific objectives, a minimum of 89 samples is required for this study.

Sampling method and subject recruitment

Formalin-fixed, paraffin-embedded (FFPE) tissues from CRC patients who underwent surgical resection of colon at Hospital USM from August 2018 till December 2020 will be obtained after approval from Hospital Director and The Human Research Ethics Committee of *USM* (*JEPeM*). The haematoxylin and eosin (H&E)-stained tissue slides were reviewed by a pathologist to ensure sufficient tumour content.

Clinicopathological and demographic data of each patient will be collected from the medical records in the hospital. This information includes:

a) Age
b) Gender
c) Race/ethnicity
d) Date of operation and colonoscopy (if available)
e) Comorbidities
f) Family history of CRC and other malignancies
g) Tumour histology
h) Tumour grade
i) Tumour site
j) AJCC Tumour Stage
k) Dukes' Staging of CRC
l) Date of last follow up
m) Date of death (if applicable)

In order to complete the sample size, archived FFPE tissue samples from our previous study, JEPeM code: (USM/JEPeM/18050239) will also be used for *KRAS* mutational status analysis after approval from Hospital Director. Details of the 34 CRC patients have been identified from previous study. Therefore, this study shall use the previous archived FFPE tissue samples as well as new FFPE tissue samples from Department of Pathology to complete the sample size number needed for significant results.

(Based on Guidelines on Retention of Pathology Records and Materials (part 1 version 1/2015))

The excess genetic material (extracted DNA) and FFPE slides (if any) will be kept at Human Genome Centre, USM until 2 years after the completion of the study. After the period, the material will be discarded. If the samples are to be used for related study after completion of this research, approval from Hospital Director will be obtained first.

Research tool

Types	Description	Owner	Location
Leica ASP300S Tissue Processor	For preparation of formalin fixed CRC tissues	Department of Pathology, USM	Department of Pathology, USM
Sakura Tissue-Tek®TEC (USA)	For embedding of the CRC tissues	Department of Pathology, USM	Department of Pathology, USM
Shandon Varistain Gemini Slide Stainer	For haematoxylin and eosin slides staining	Department of Pathology, USM	Department of Pathology, USM
NanoQuant Infinate M200 (TECAN, Switzerland) spectrometer	For quantification of the extracted DNA	Human Genome Centre, School of Medical Sciences, USM	Human Genome Centre, School of Medical Sciences, USM
Agilent SureCycler 8800 Thermal Cylcer	For amplification of targeted gene	Human Genome Centre, School of Medical Sciences, USM	Human Genome Centre, School of Medical Sciences, USM
BigDye® Terminator v3.1 cycle sequencing kit	For gene sequencing	First Base Laboratories Sdn. Bhd. (Malaysia)	No. 7-1 to 7-3, Jalan SP 2/7, Taman Serdang Perdana, Seksyen 2, 43300 Seri Kembangan, Selangor, Malaysia

Operational definition

Depending on the *KRAS* mutational analysis results, subjects are classified as either having:

a) Wild type *KRAS* (no *KRAS* mutations at codon 12 or 13) or
b) Mutant *KRAS* (presence of *KRAS* mutations at codon 12 or 13).

Data collection method

Subject recruitment and DNA extraction:

Subjects will include CRC patients that underwent surgical resection of CRC in which the FFPE tissues are stored in the Department Pathology, as consented by the patients. Archive FFPE tissues in Human Genome Centre from previous study will also be used after ethical approval. For DNA extraction, 10 µm thick sections from the FFPE tissue samples will be used for each case. Pathologist and laboratory technologist from Department of Pathology will be responsible in preparation of tissue slides with sufficient tumour content for each subject enrolled in this study. Then, DNA will be extracted from these tissues using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions at Human Genome Centre, USM. Quantity and quality of the extracted DNA will be analysed using spectrophotometer and agarose gel electrophoresis respectively. DNA samples will be kept at 4^{o}C until further analysis. To ensure patient's confidentiality, the DNA samples will be labelled with anonymous ID (e.g., CRC1). The process of extracting DNA and further analysis described below will be performed by MPath student, responsible for this current study.

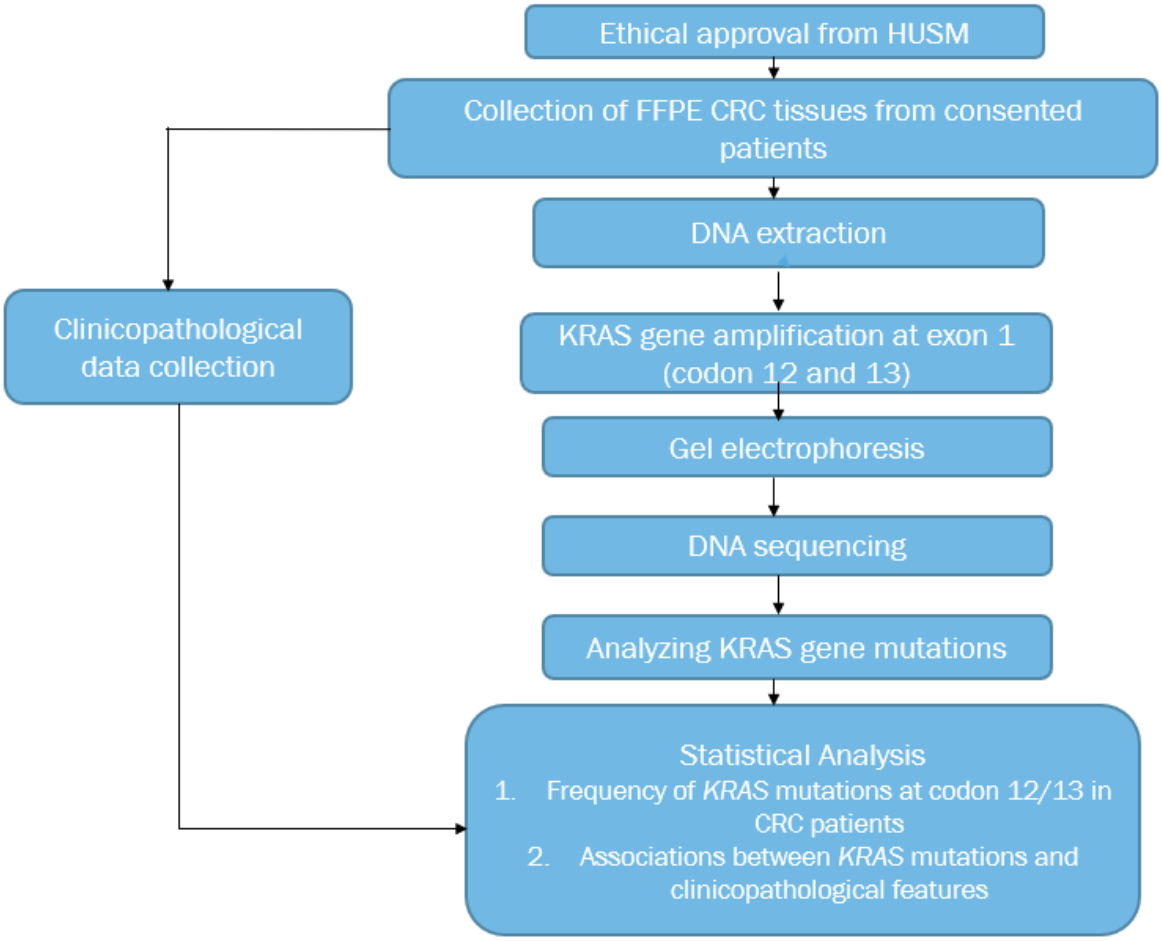
Detection of mutations in KRAS gene:

Exon 1 of *KRAS* encompassing codon 12 and 13 will be amplified by polymerase chain reaction (PCR) using previously published primers sequences, cycling conditions and annealing temperatures (Yip et al., 2013). The forward primer (5' – 3') sequence that will be used is ACCTTATGTGTGCATGTTC while the reverse primer (5'- 3') is CTATTGTTGGATCATATTCG. The planned cycling condition includes predenaturing incubation at 95^{o}C for 5 minutes, 35 cycles of 94^{o}C for 1 minute, annealing temperature of 53^{o}C for 1 minute, and 72^{o}C for 30 seconds followed by a final extension at 72^{o}C for 5 minutes. Each PCR reaction contain 30 ng genomic DNA, 1.5mM $MgCl_2$, 0.25 mM of each deoxynucleotide triphosphate (dNTP), 0.5 µM of each primer and Taq DNA polymerase, in a final reaction volume of 20 µL.

DNA Sequencing:

The PCR product will be purified using QIAquick PCR purification kit (QIAGEN, Germany) according to the manufacturer's instruction. 20 µL of PCR product will be mixed with 100 µL PB buffer and transferred into QIAquick column. The column will be centrifuged at 13000 rpm for 30 seconds and the flow through out from the column will be discarded. Next, 750 µL of PE buffer will be added into the column before being centrifuged at 13000 rpm for 30 seconds. Finally, 20 µL of buffer EB will be added to elute the DNA. The final 20 µL purified PCR product will be sent to First Base Laboratories Sdn. Bhd. (Malaysia) for sequencing analysis. All possible somatic mutations at codon 12 and 13 from exon 1 of *KRAS* gene are expected to be identified using this technique.

Study flowchart



Data Analysis

Statistical analysis will be performed using SPSS version 24.0. The association between each clinicopathological features and the status of *KRAS* gene mutations in the tissue will be analysed using Chi-Square test. All p values will be estimated using two-sided statistical test. A p-value of <0.05 is considered statistically significant.

Expected result(s)

The percentage of patients with wild type and mutant *KRAS* will be calculated and their clinicopathological features will be compared and analysed to demonstrate any association between the two variables. The frequency of the type of point mutations at codon 12 and 13 will be represented in a table to demonstrate the most frequent mutations observed among the patients.

The results can be presented as in Table 1 and 2 below:

Table 1: Association of clinicopathological features with *KRAS* mutational status

Clinicopathological feature	**Wild type *KRAS* n = (%)**	**Mutant *KRAS***		**P value**
		Codon 12 n = (%)	Codon 13 n = %	
Age (median, range)				
Gender Male Female				
Race/Ethnicity Malay Chinese Indian				
T stage T1 or T2 T3 or T4				
N stage N0 N1 or N2 N3				
M stage M0 M1				
Duke's staging Duke's A&B Duke's C Duke's D				
Histology grade Well/ moderately differentiated Poorly differentiated				
Tumour location Right side Left side				

Table 2: Frequency and type of *KRAS* mutations

***KRAS* somatic mutation**	**n (%)**
ex. G12A	
G12D	
G12C	
G12V	
G13D	

Gantt Chart and Milestone

Gantt Chart

Activity	2021												2022											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Ethical approval																								
Collection of samples and clinicopathological data of subjects																								
DNA extraction																								
***KRAS* gene amplification**																								
DNA sequencing																								
Data analysis																								
Writing and submission of report																								

Milestone

Activity	Start date	Completion date
Approval for ethical application	1st January 2021	31st March 2021
Collection of samples and clinicopathological features of subjects	1st April 2021	31st January 2022
DNA extraction from FFPE tissues	1st May 2021	31st March 2022
***KRAS* gene amplification**	1st July 2021	30th April 2022
DNA Sequencing	1st February 2022	30th June 2022
Data Analysis	1st May 2022	31st October 2022
Writing and submission of report	1st September 2022	31st December 2022

Ethical consideration(s) [if applicable]

1. **Subject vulnerability**
   Subjects recruited were given full freedom to agree or to refuse in collection of their tissue samples for study purposes without losing the benefits of standard clinical care. The samples were collected and stored accordingly as consented by the patients.

2. **Declaration of absence of conflict of interest**
   There will be no conflict of interest between the parties involved in this study.

3. **Privacy and confidentiality**
   Patients' medical information will be kept confidential by the study doctor and staff and will not be made publicly available unless disclosure is required by the law. Data obtained from this study that does not identify patient individually will be published for knowledge purposes. Patients' original medical records may be reviewed by the researcher, the Ethical Review Board for this study, and regulatory authorities for the purpose of verifying the clinical data. Patients' medical information may be held and processed on a password-protected computer.

   Excess genetic material (extracted DNA) and FFPE slides (if any) will be kept at Human Genome Centre, USM until 2 years after the completion of the study and to be used for related study under the approval of The Human Research Ethics Committee of *USM* (*JEPeM*).

4. **Community sensitivities and benefits**
   The finding of this study may benefit the community by increasing our understanding of the genetic profile of CRC patients in Kelantan. This information may serve as a preliminary study for further research exploring the susceptibility genes in development of CRC in a bigger sample population in Kelantan. It is our hope that in the future, the outcome of this study may contribute to the improvement in detection, management and treatment of patients diagnosed with CRC.

5. **Honorarium and incentives**
   This study will not involve any honorarium given to the subjects. No charge will be incurred on the patients. A token of appreciation will be given to the technologists who are involved in the preparation of the FFPE tissues and H&E tissue slides.

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2.2 ETHICAL APPROVAL LETTER

Jawatankuasa Etika
Penyelidikan Manusia USM (JEPeM)

Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
Kampus Kesihatan
16150 Kubang Kerian, Kelantan. Malaysia.
Tel. : +609 - 767 3000/2354/2362
Fax. : + 609 - 767 2351
Email : jepem@usm.my
Laman Web : www.jepem.kk.usm.my
www.usm.my

6th July 2021

Dr. Hidayati Husainy Hasbullah
Human Genome Centre
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM Code : USM/JEPeM/21030249
Protocol Title: KRAS Gene Mutational Profiles among Colorectal Cancer (CRC) Patients in Hospital Universiti Sains Malaysia.

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/21030249**, which should be used for all communications to JEPeM-USM in relation to this study. This ethical approval is valid from **6th July 2021** until **5th July 2022.**

Study Site: Hospital Universiti Sains Malaysia.

The following researchers are also involved in this study:

1. Dr. Marahaini Musa
2. Assoc. Prof. Dr. Sarina Sulong
3. Dr. Nur Asyilla Che Jalil

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the above mentioned document, the following technical documents were included in the review on which this approval was based:

1. Data Collection Sheet (Demographic Data)

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2019: Continuing Review Application Form.**
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using **JEPeM-USM FORM 3(A) 2019: Study Protocol Amendment Submission Form.**
3. Revisions in the informed consent form using the **JEPeM-USM FORM 3(A) 2019: Study Protocol Amendment Submission Form.**
4. Reports of adverse events including from other study sites (national, international) using the **JEPeM-USM FORM 3(G) 2019: Adverse Events Report.**
5. Notice of early termination of the study and reasons for such using **JEPeM-USM FORM 3(E) 2019.**
6. Any event which may have ethical significance.
7. Any information which is needed by the JEPeM-USM to do ongoing review.
8. Notice of time of completion of the study using **JEPeM-USM FORM 3(C) 2019: Final Report Form.**

JEPeM
JAWATANKUASA ETIKA PENYELIDIKAN MANUSIA

Please note that forms may be downloaded from the JEPeM-USM website: **www.jepem.kk.usm.my**

JEPeM-USM is in compliance with the Declaration of Helsinki, International Conference on Harmonization (ICH) Guidelines, Good Clinical Practice (GCP) Standards, Council for International Organizations of Medical Sciences (CIOMS) Guidelines, World Health Organization (WHO) Standards and Operational Guidance for Ethics Review of Health-Related Research and Surveying and Evaluating Ethical Review Practices, EC/IRB Standard Operating Procedures (SOPs), and Local Regulations and Standards in Ethical Review.

Thank you.

Sincerely

ASSOC. PROF. DR. AZLAN HUSIN
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia) JEPeM
Universiti Sains Malaysia