UNIVERSITI PUTRA MALAYSIA

ANALYSIS OF V-KI-RAS2 KIRSTEN RAT SARCOMA VIRAL ONCOGENE (KRAS) AND DIFFERENTIAL EXPRESSED MIRNA IN VARIOUS STAGES OF COLORECTAL CANCER

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

ANALYSIS OF V-KI-RAS2 KIRSTEN RAT SARCOMA VIRAL ONCOGENE (KRAS) AND DIFFERENTIAL EXPRESSED MIRNA IN VARIOUS STAGES OF COLORECTAL CANCER

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June 2013

Chair : Assoc. Prof. Cheah Yoke Kqueen

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Colorectal cancer is one of the most prevalent cancers in Malaysia. There are many factors that contribute towards colorectal carcinogenesis and one of them is genetic predisposition. Mutations in the V-Ki-Ras2 (Kras) oncogene have been implicated in 30-50% of the colorectal cancer patients and usually lead to significantly poorer prognosis. Early detection of colorectal cancer still poses a huge problem to the clinicians as there are few or no signs at all during the initial phase of colorectal cancer. However, recently a group of small, non-coding RNAs called microRNAs (miRNAs) was discovered to play a role in colorectal carcinogenesis. Therefore, the aim for this study is to elucidate and investigate the Kras oncogene and miRNAs expression level in various stages of colorectal cancer for better understanding of this disease. In this study, colorectal cancer and adjacent normal tissue samples were obtained from Hospital Kuala Lumpur. The study was divided into 2 parts. The first part was Kras mutational studies using PCR-RFLP method and the second part was next-generation sequencing and differential expression analysis on paired cancer and

normal samples. The results obtained showed that Kras mutation was at 39%. From the sequencing results, approximately 92% clean reads were obtained and length distribution showed that the small RNA ranges from 20-28 nucleotides. This study also managed to identify 22 differentially expressed miRNAs and 5 of them were chosen for further validation using real-time PCR. Three of the miRNAs that were up-regulated are miR-106a, miR-135b and miR-21, while miR-1 and miR-504 were down-regulated. Further evaluation using *in silico* analysis managed to identify putative targets for the miRNAs. Among the interesting findings was miR-135b targets APC gene that is involved in the Wnt signalling pathway, which is among the crucial pathways during initiation of colorectal carcinogenesis. Besides, miR-1, miR-106a and miR-21 were predicted to regulate genes involved in EGFR and Kras signalling pathways. Meanwhile, one of the putative targets for miR-504 was BCL_2 gene which regulates the p53 tumor suppressor gene. Although this is only a preliminary profiling study, the results obtained can provide some insights on the role of Kras oncogene and miRNAs on colorectal carcinogenesis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISIS V-KI-RAS2 KIRSTEN RAT SARCOMA VIRAL ONCOGENE (KRAS) DAN EKSPRESI MIRNA YANG BERBEZA DALAM KANSER KOLOREKTAL BERLAINAN PERINGKAT

Oleh

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Pengerusi : Prof. Madya Cheah Yoke Kqueen

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Kanser kolorektal merupakan salah satu kanser yang paling kerap di Malaysia. Terdapat banyak faktor yang menyumbang kepada proses karsinogenesis kolorektal dan salah satu daripadanya ialah kecenderungan genetik. Mutasi dalam onkogen V-Ki-Ras2 (Kras) telah dikenalpasti dalam 30-50% pesakit kanser kolorektal dan selalunya mendorong kepada prognosis yang lebih teruk. Pengesanan awal kanser kolorektal masih lagi menjadi masalah kepada para doctor kerana kanser kolorektal selalunya tidak menunjukkan kesan yang ketara di awal peringkat penyakit ini. Kebelakangan ini, sekumpulan RNA kecil yang dipanggil mikroRNA (miRNA) telah didapati memainkan peranan dalam proses karsinogenesis kolorektal. Oleh itu, objektif penyelidikan ini adalah untuk mengkaji dan menyelidik ekspresi dan fungsi onkogen Kras dan miRNA dalam pelbagai peringkat kanser kolorektal untuk lebih memahami penyakit ini. Dalam kajian ini, sampel tisu kanser kolorektal dan tisu normal diambil daripada Hospital Kuala Lumpur. Penyelidikan ini dibahagi kepada 2 bahagian. Pertama ialah kajian mutasi Kras menggunakan kaedah PCR-RFLP,

manakala bahagian kedua ialah proses penjujukan generasi akan datang (NGS) dan analisis ekspresi berbeza terhadap pasangan sampel kanser dan normal. Hasil kajian ini menunjukkan mutasi Kras adalah 39%. Keputusan penjujukan pula menunjukkan hasil bacaan bersih lebih kurang 92% dan distribusi panjang RNA kecil berada dalam lingkungan 20-28 nukleotida. Kajian ini juga menemui 22 miRNA yang menunjukkan ekspresi berbeza dan 5 daripadanya dipilih untuk validasi selanjutnya menggunakan PCR masa nyata. Tiga daripada miRNA tersebut menunjukkan peningkatan regulasi iaitu miR-106a, miR-135b dan miR-21, manakala miR-1 dan miR-504 menunjukkan penurunan regulasi. Penilaian selanjutnya menggunakan analisis *in silico* berjaya mengenal pasti sasaran putative bagi miRNA. Antara penemuan yang menarik ialah miR-135b mensasar gen APC yang terlibat dalam rangkaian signal Wnt yang merupakan salah satu rangkaian penting semasa permulaan karsinogenesis kolorektal. Selain itu, miR-1, miR-106a dan miR-135b dijangka memainkan peranan dalam regulasi gen-gen di dalam rangkaian signal EGFR dan Kras. Di samping itu, salah satu gen sasaran miR-504 ialah BCL_2 yang berperanan dalam regulasi gen pembantut tumor p53. Walaupun kajian ini adalah hanya satu kajian profil awal, keputusan yang diperolehi boleh memberikan beberapa perspektif mengenai peranan onkogen Kras dan miRNA dalam karsinogenesis kolorektal.

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I certify that a Thesis Examination Committee has met on 13 June 2013 to conduct the final examination of Lye Kwan Liang on his thesis entitled "Analysis of V-Ki-Ras2 Kirsten Rat Sarcoma Viral Oncogene (KRAS) and Differential Expressed miRNA in Various Stages of Colorectal Cancer" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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LYE KWAN LIANG

Date: 13 June 2013

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LIST OF ABBREVIATIONS

5-FU	:	5-Fluorouracil
AJCC	:	American Joint Committee on Cancer
APC	:	Adenomatous Polyposis Coli
ASR	:	Age-standardized Incidence Rate
BGI	:	Beijing Genomic Institute
cDNA	:	Complementary Deoxyribonucleic Acid
ChiP-seq	:	Chromatin Immunoprecipitation-sequencing
CLL	:	Chronic Lymphocytic Leukemia
CRC	:	Colorectal Cancer
DNA	:	Deoxyribonucleic Acid
EGFR	:	Epidermal Growth Factor Receptor
EtBr	:	Ethidium Bromide
FAP	:	Familial Adenomatous Polyposis
FFPE	:	Formaldehyde Fixed-paraffin Embedded
GAPs	:	GTPase Activating Proteins
GDP	:	Guanosine Diphosphate
GEFs	:	Guanine Nucleotide Exchange Factors
GTP	:	Guanosine Triphosphate
HKL	:	Hospital Kuala Lumpur
HNPCC	:	History of Inflammatory Bowel Disease
HUKM	:	Hospital Universiti Kebangsaan Malaysia
KEGG	:	Kyoto Encyclopedia of Genes and Genomes
MAPK	:	Mitogen-activated Protein Kinase
MiRNAs	:	MicroRNAs
MREC	:	Medical Research Ethics Committee

mRNA	:	Messenger RNA
NCBI	:	National Center for Biotechnology Information
NGS	:	Next-Generation Sequencing
NMRR	:	National Medical Research Registry
PAGE	:	Polyacrylamide Gel Electrophoresis
PCR	:	Polymerase Chain Reaction
PI-3-k	:	Phophatidylinositol-3-kinase
Pre-miRNA	:	Precursor MiRNA
Pri-miRNA	:	Primary MiRNA
qPCR	:	Quantitative Real-time Polymerase Chain Reaction
RB1	:	Retinoblastoma 1
RFLP	:	Random Fragment Length Polymorphism
RIN	:	RNA Integrity Number
RISC	:	RNA-induced Silencing Complex
RNA	:	Ribonucleic Acid
ROS	:	Reactive Oxygen Species
rRNA	:	Ribosomal Ribonucleic Acid
scRNA	:	Small Cytoplasmic Ribonucleic Acid
snoRNA	:	Small Nucleolar Ribonucleic Acid
SNPs	:	Single Nucleotide Polymorphisms
TGFBR2	:	Transforming Growth Factor-B Receptor 2
TPM	:	Transcript Per Million
tRNA	:	Transfer Ribonucleic Acid
UTRs	:	Untranslated Regions

CHAPTER 1

INTRODUCTION

1.1 Background of study

Colorectal cancer is among the most prevalent cancer in Malaysia. According to the Second Report of the National Cancer Registry, colorectal cancer was ranked as the first and third most common cancer in men and women respectively (Gerald and Halimah, 2004). In a more recent data obtained from the National Cancer Registry Report 2007, colorectal cancer was placed second as the leading cancers among population in Malaysia. The age-standardized incidence rates (ASR) were 85.1 per 100000 males and 94.4 per 100000 females. Globally, colorectal cancer is ranked as third most common diagnosed cancer in males and second in females (Jemal et al., 2010). This indicates that colorectal cancer is affecting and probably will affect more and more people. Early diagnosis of colorectal cancer can often lead to a complete cure. However, most of the cases were usually detected at a more advanced stage, making the cancer harder to treat. This is due to the fact that colorectal cancer exhibits few or no signs at all during the early stages of the disease. The lifetime risk of getting colon cancer in developed countries is approximately 5% and is stable or decreasing (Bretthauer, 2010). However, in developing countries, the rate continues to escalate due to increase exposure to risk factors (Edwards et al., 2010).

Majority of colorectal cancer are sporadic, with hereditary cases only contributing approximately 10-15% of the total cases. There are many known factors for colorectal cancer which includes modifiable and non-modifiable risk factors. Among the modifiable risk factors are smoking, physical inactivity, overweight and obesity, red and processed meat consumption, and excessive alcohol consumption. The non-modifiable risk factors include age, inherited genetic risk such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) and history of inflammatory bowel disease (Haggar and Boushey, 2009).

There are a few oncogenes linked to colorectal cancer and *ras* family is one of the well studied and characterized one. There are 3 known isoforms of this family, Nras, Hras and Kras. They played an important role in cell metabolism including cell proliferation, apoptosis, migration and differentiation. Among the isoforms, approximately 90% of *ras* mutation comprised of Kras. Besides, Kras was also found to be mutated in 21.6% of all human cancers and 30-50% in colorectal cancers (Vogelstein et al., 1988, Andreyev et al., 1998). The Kirsten Ras In-Colorectal-Cancer Collaborative Group (RASCAL) reported that presence of Kras mutation was significantly associated with poorer prognosis. In the same study, codon 12 mutation was found in 27.7% of the patients, while codon 13 mutation was found in 6.6% of the patients. Kras mutation was also associated with poorer response to anti-EGFR therapy, and in some cases even detrimental to the patients (Bokemeyer et al., 2011).

MicroRNAs (miRNAs) are transcript of 19-25 nucleotides and are encoded in the genomes of vertebrates, invertebrates and plants (Ambros, 2004). Recent findings

have shown that miRNAs played a pivotal role in crucial processes such as cell proliferation, apoptosis, development, differentiation and metabolism (Yang et al., 2009). MiRNAs will bind to the 3'-untranslated regions (UTRs) of their target mRNAs to regulate gene expression. They will stop transcription if the target is in perfect complementary, and stop translation if there is only partial complementary (Slaby et al., 2007). Consistent with translational control, miRNAs will reduce the protein levels of target genes but barely affects the mRNA levels of these genes. The classical view of molecular oncology states that cancer is a genetic disease involving tumor suppressors and oncogenic proteins (Negrini et al., 2009). However, lately it has been discovered that miRNAs are also involved in human tumorigenesis. The first evidence that miRNAs are involved in cancer came from the finding that miR-15a and miR-16-1 are down-regulated or deleted in most patients with chronic lymphocytic leukemia (CLL) (Calin et al., 2002). Meanwhile, a few miRNAs were found to be associated with colorectal cancer such as the miR-17-92 cluster, miR-21, miR-34, miR-135 and miR-196a (Zhu et al., 2008; Schimanski et al., 2009; Rossi et al., 2009; Kim et al., 2009).

1.2 Problem Statement

Colorectal cancer is among the most prevalent cancer in Malaysia and also the world. Many studies had been carried out to better understand this cancer in order for a better diagnosis and prognosis. Early diagnosis of colorectal cancer often leads to complete cure. However, early detection still poses a huge problem to us as colorectal cancer exhibit few or no signs at all during the initial phase. The gold standard for prognosis still depends on the tumor staging at the time of diagnosis. Recently, miRNAs had shown some promising results in cancer research. Many miRNAs were found to play a major role in regulatory mechanism involve in numerous cancers. Differential miRNAs expression between tumors and healthy controls are useful in distinguishing and identifying miRNAs that could play an important role in carcinogenesis. However, the differential miRNAs profile in different stages of colorectal cancer is still unavailable for Malaysia population. Therefore, we are interested to study the Kras oncogene and differential miRNAs expression level in various stages of colorectal cancer patients in Malaysia population for potential biomarkers and therapeutic targets development.

1.3 Research objectives

1.3.1 General objective

The general objective of this study is to elucidate and investigate the Kras oncogene and differential miRNAs expression level in various stages of colorectal cancer.

1.3.2 Specific objectives

- To profile Kras oncogene and differential miRNAs expression level in different stages of colorectal cancer
- To investigate the relationship between demographic data, miRNAs expression level and Kras mutations with different stages of colorectal cancer.
- To use next generation sequencing technique to profile and identify differentially expressed miRNAs in colorectal cancer and adjacent normal tissue

1.4 Hypotheses

- Kras oncogene mutational status and miRNAs expression profile can be obtained for the colorectal cancer patients among Malaysian population
- Age, gender and ethnicity does correlate with certain stages of colorectal cancer
- Next generation sequencing technique is able to robustly profile and identify differentially expressed miRNAs in colorectal cancer and adjacent normal tissue

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