GLOBAL MICRORNA GENE EXPRESSION PROFILES ANALYSIS AND POSSIBLE ROLE OF MICRORNA-5P AND 3P PAIRS IN HUMAN COLON CANCER CELLS

SOON YUEN LOON

MASTER OF MEDICAL SCIENCES

FACULTY OF MEDICINE AND HEALTH SCIENCES
UNIVERSITI TUNKU ABDUL RAHMAN
SEPTEMBER 2013

GLOBAL MICRORNA GENE EXPRESSION PROFILES ANALYSIS AND POSSIBLE ROLE OF MICRORNA-5P AND 3P PAIRS IN HUMAN COLON CANCER CELLS

By

SOON YUEN LOON

A dissertation submitted to Faculty of Medicine and Health Sciences,
Universiti Tunku Abdul Rahman,
in partial fulfilment of the requirements for the degree of
Master of Medical Sciences
September 2013

ABSTRACT

GLOBAL MICRORNA GENE EXPRESSION PROFILES ANALYSIS AND POSSIBLE ROLE OF MICRORNA-5P AND 3P PAIRS IN HUMAN COLON CANCER CELLS

Soon Yuen Loon

Colon cancer is the second most prevalent cancer-related death in Malaysia. Previous studies have revealed involvement of microRNA (miRNA) in colon carcinogenesis. A miRNA precursor may generate alternative species (derived from 5p or 3p of the precursor) targeting at different genes. In most cases, only one species, either 5p or 3p-derived miRNA, remains while the other is degraded. However, recent reports have demonstrated co-expression of some functional miRNA-5p and -3p species contribute to disease progression. To systematically identify differentially expressed miRNAs in colon cancer cells, four colon cancer cell lines were subjected to global miRNA gene expression profiling analysis, followed by bioinformatics and statistical analysis. Selected miRNAs data were validated using real-time qRT-PCR. Furthermore, putative targets of selected miRNAs and associated biological functions correlating with respective pathways were predicted using bioinformatics algorithms. In the analysis, 128 miRNAs (10.8%) were revealed to be differentially expressed in colon cancer cells relative to normal tissues. Among them, 92 miRNAs (71.9%) were up-regulated and 36 (28.1%) were down-regulated, suggesting significant miRNA deregulation in colon carcinogenesis. Hierarchical clustering analysis demonstrated separation of

normal from cancer cells. Nineteen miRNA-5p/-3p pairs (38 miRNAs) were significantly co-expressed in colon cancer cells indicating the biological significance of both species. The target genes of the nineteen miRNA-5p/-3p pairs were found involved in specific cancer-related pathways: 42.1% are epithelial-mesenchymal transition (EMT)-related, 42.1% are p53 signalling pathway-related, 55.3% are angiogenesis-related and 71.1% are apoptosis-related. To experimentally validate differential targeting by miRNA-5p/-3p pair, let-7d-5p and -3p and their respective putative targets, *IGF1R* and *KRAS*, which are both associated with colon cancer, were tested. To achieve miRNA over-expression or knockdown, HCT-15 cells were transiently transfected with let-7d-5p inhibitor or let-7d-3p mimic, respectively. Real-time qRT-PCR and western blot quantification results supported differential targeting of the two transcripts by let-7d-5p and -3p. In conclusion, a miRNA profile of colon cancer cells was established which had led to the finding of frequent miRNA-5p and -3p pairs co-expression in colon cancer cells. A co-expression miRNA pair and their putative targets were experimentally confirmed suggesting the miRNA pair could be functionally relevant to colon carcinogenesis.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to my supervisors Prof. Choo Kong Bung and Assoc. Prof. Dr. Alan Ong Han Kiat for their patience, guidance and encouragement throughout my study and thesis writing.

I wish to acknowledge the valuable advices and supports from Prof Dr. Huang Chiu-Jung from Chinese Culture University, Taiwan; Prof. Yap Sook Fan, Prof. Shelly Soo, Assoc. Prof. Dr. Lim Yang Mooi, Assoc. Prof. Dr. Gan Seng Chiew and Dr. Leong Pooi Pooi from Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman (UTAR). Special thanks to lab seniors and colleagues Lai Kuan, Kian Lam, Ying Ni, Han Chung, Hoon Koon, Teck Yew, Siak Mun, Shao Feng, Yu Siong and Shu Cheow.

I would like to thank Malaysian Ministry of Higher Education (MOHE) for providing me MyMaster sponsorship and also UTAR research fund for supporting this study. Lastly, I would like to thank my family members for their encouragement and support.

APPROVAL SHEET

This dissertation entitled "**GLOBAL MICRORNA GENE EXPRESSION PROFILES ANALYSIS AND POSSIBLE ROLE OF MICRORNA-5P AND 3P PAIRS IN HUMAN COLON CANCER CELLS"** was prepared by SOON YUEN LOON and submitted as partial fulfilment of the requirements for the degree of Master of Medical Sciences at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Prof. Dr. Choo Kong Bung) Date: ……..
Professor/ Supervisor
Department of Pre-clinical Sciences
Faculty of Medicine and Health Sciences
Universiti Tunku Abdul Rahman

_____

(Assoc. Prof. Dr. Alan Ong Han Kiat) Date: ……..
Associate Professor/ Co-supervisor
Department of Pre-clinical Sciences
Faculty of Medicine and Health Sciences
Universiti Tunku Abdul Rahman

FACULTY OF MEDICINE AND HEALTH SCIENCES

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

SUBMISSION OF DISSERTATION

It is hereby certified that **Soon Yuen Loon** (ID No: **12UMM02207**) has completed this dissertation entitled "GLOBAL MICRORNA GENE EXPRESSION PROFILES ANALYSIS AND POSSIBLE ROLE OF MICRORNA-5P AND 3P PAIRS IN HUMAN COLON CANCER CELLS" under the supervision of Prof. Dr. Choo Kong Bung (Supervisor) and Associate Prof. Dr. Alan Ong Han Kiat (Co-supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences.

I understand that University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(Soon Yuen Loon)

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Name _____

Date _____

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3’-UTR	3’-untranslated region
APS	Ammonium persulfate
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
$\Delta\Delta C_T$	Comparative cycle threshold
CEA	Carcinoembryonic antigen
CRC	Colorectal cancer
CSAp	Colon specific antigen-p
C_T	Cycle threshold
CTC	Computed tomography colonography
DMEM	Dulbecco's Modified Eagle Medium
DMNT3A	DNA methyltransferase 3A
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescent
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
EMT	Epithelial-mesenchymal transition
ERK5	Extracellular signal-regulated kinase 5
FBS	Foetal bovine serum

FFL	Feed-forward loop
FOBT	Faecal occult blood test
HCA	Heterocyclic amines
HRP	Horseradish peroxidase
IGF1R	Insulin-like growth factor 1 receptor
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Medium
MET	Mesenchymal-to-epithelial transition
MiRNA	microRNA
MMP2	Metalloproteinase 2
Mrna	Messenger RNA
NaF	Sodium fluoride
NCBI	National Center for Biotechnology Information
NEM	N-Ethylmaleimide
PAH	Polycyclic aromatic hydrocarbon
PI3K	Phosphatidylinositol-3-kinase
PMSF	Phenylmethylsulfonyl fluoride
qRT-PCR	Real-time quantitative reverse transcription PCR
RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween-20
TCA cycle	Tricarboxylic acid cycle
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TGFβ	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

Colon cancer is a disease initiated from abnormal and uncontrolled cell division and growth in colon, forming a growing mass of cancer cells. According to global cancer statistic GLOBOCAN 2008, colon cancer is the third most frequent cancer worldwide (Ferlay et al., 2010; Bray et al., 2013). Approximately one million new cases are reported annually with an estimated mortality of more than half a million death (Wu et al., 2011). In Malaysia, colon cancer is emerging as the second most frequently diagnosed cancer and the second leading cause of cancer mortality regardless of sex (Ariffin and Saleha, 2011), with a prevalence which is even higher than the worldwide statistics. The National Cancer Registry of Malaysia has reported that colon cancer is slightly more common in males and in the Chinese ethnic group in Malaysia (Ariffin and Saleha, 2011). National Cancer Institute of U.S. and the National Cancer Society Malaysia have listed the risk factors that may increase the chances of developing colon cancer include: ageing, polyps, sedentary lifestyle, unhealthy eating habits, smoking, family history, inherited genetic conditions (Familial adenomatous polyposis and Lynch syndrome) and ulcerative or Crohn colitis.

The pathogenesis of colon cancer involves step-wise progression starting from benign adenoma (polyps) which may take decades to develop into invasive adenocarcinoma (Nguyen et al., 2009). Once the invasive stage is reached, the malignant colon cancer cells rapidly spread, infiltrate and colonise distant organs, such as the liver, to become a metastatic cancer (Alberici, 2007; Nguyen et al., 2009; Wu et al., 2011). For this reason, screening and early diagnosis of colon cancer are highly recommended to ease the disease burden and to improve the survival rate (Geiger and Ricciardi, 2009; Al-Naggar and Bobryshev, 2013), highlighting the necessity of unique and specific diagnostic cancer markers and screening tools.

In recent years, non-coding genomic sequences, including small nucleolar RNA, small interfering RNA (siRNA) and miRNA have been revealed to be involved in the carcinogenesis process (Galasso et al., 2010). Of these noncoding RNAs, a remarkable number of miRNAs have been shown to exhibit differential expression in various cancer tissues, including colon cancer, which alter cell proliferation, apoptosis and metastasis through interactions with intracellular signalling networks (Galasso et al., 2010; Liu et al., 2011a; Wu et al., 2011). Polymorphisms within miRNA binding sites in cancer-related genes such as *CD86* and *INSR*, are associated with higher risk for colon cancer (Landi et al., 2008; Wu et al., 2011). Furthermore, miRNA dysregulation or polymorphisms of miRNA target transcript are reported to show correlation with patient survival or disease prognosis of several cancer types, including colon cancer (Christensen et al., 2009; Wu et al., 2011;

Cascione et al., 2013). Further investigation into dysregulation of miRNA in colon cancer, together with the development of RNA delivery technology, potential novel miRNA screening tools and miRNA-based therapeutics are anticipated.

In most cases, only one strand, either derived from the 5p or 3p arm, of the RNA duplex is selected to enter and assemble with the RNA-induced silencing complex (RISC), and is consequently expressed in abundance, while the alternative strand, known as passenger strand or miRNA* strand, is degraded (Alexiou et al., 2010; Guo and Lu, 2010; Yang et al., 2011). The selection as to which strand is incorporated into RISC is influenced by the difference in thermodynamic stabilities between the two ends of the miRNA:miRNA* duplex (Ruby et al., 2007; Alexiou et al., 2010; Guo and Lu, 2010; Barrero et al., 2011). Functional miRNAs usually have less stable 5' ends that preferentially leads to incorporation of the 5' end into the RISC and, this results in efficient mRNA targeting and degradation (Schuck et al., 2004; Barrero et al., 2011).

However, recent reports have demonstrated abundant co-expression of both miRNA strands, including the miRNA* strand, and both co-expressed miRNAs are functional and contribute to disease progression, including colon cancer (Jazdzewski et al., 2009; Guo and Lu, 2010; Jiang et al., 2010; Lin et al., 2010; Jin et al., 2011; Almeida et al., 2012). In the case where the

miRNA* strand was reported as the abundant mature miRNA, miR-#-5p (from 5’ arm) or miR-#-3p (from 3’ arm) was denoted. And the miRNA-5p and 3p species have different sequences, they target different transcripts sets. Guo and Lu (2010) have reported that well-conserved miRNA* strands in seed sequences may contribute to network regulation in cellular processes.

Many research have demonstrated that cancer cells have differential miRNA expression profiles relative to normal cells in various cancers, highlighting the diagnostic and therapeutic potential of miRNAs in cancer detection and treatment (Paranjape et al., 2009; Wu et al., 2013). Analyses of miRNAs in mechanistic studies are crucial for a better understanding of colon cancer pathogenesis of at early stages and for eventual identification of novel biomarkers and therapeutic targets with fewer side effects for better prognosis. Besides, miRNA expression profiles can correctly classify human cancers of unknown primary origin as well as of poorly differentiated tumours (Lu et al., 2005; Calin and Croce, 2006). Therefore, the elucidation of the molecular mechanism of the pathogenesis of colon cancer enables us to understand miRNA dysregulation and their cancer-related phenotypic functions in the disease, and provides a better and earlier clinical diagnosis and prognosis mean for colon cancer patients.

We hypothesise that both miRNA-5p and -3p are co-dysregulated in colon cancer cells and miRNA dysregulation of 5p and 3p species are

associated with alterations of expression of miRNA targeted transcripts in important cancer pathways in colon carcinogenesis. These epigenetic changes and the affected gene expression changes in the cancer cells may help to elucidate crucial steps in colon carcinogenesis.

The objectives developed for this study were:-

1) To identify differentially expressed miRNAs in colon cancer cells relative to normal cells by high-throughput miRNA real-time PCR array
2) From the real-time PCR array results, to identify and focus on the dysregulated miRNA-5p and -3p pairs in colon cancer cells
3) To analyse the expression pattern of the miRNA-5p and -3p pairs to determine if they are co-up or co-down-regulated
4) To identify and map putative targets for selected dysregulated miRNAs pairs by bioinformatics approaches
5) To validate putative targets of a cancer-related regulatory pathway of a selected miRNA pair in colon cancer cells by miRNA transfection assays

CHAPTER 2

LITERATURE REVIEW

2.1 Colon Cancer

Colon cancer, together with rectal cancer, is often jointly known as colorectal cancer (CRC) or large bowel cancer. According to the global cancer statistic GLOBOCAN 2008, colon cancer is the third most commonly diagnosed cancer in men, the second most common in women (Ferlay et al., 2010; Bray et al., 2013). As stated by "World Health Organization" (2012), colon cancer has become the fourth leading cause of cancer death in both genders worldwide. Colon cancer is often recurred in patients with advanced stage of cancer as current available treatments are unable to fully eradicate the cancer cells though colon cancer is generally well understood from a genetic perspective (O'Brien et al., 2007). The recurrence of colon cancer and cancer chemo-drug resistance could be partially due to another recently discovered rare cell population in the tumour - colon cancer stem cells (Caruso et al., 2012; Puglisi et al., 2013). This cell subpopulation is probably responsible for tumour formation and maintenance due to their self-renewal and multi-lineage differentiation properties (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Seidel et al., 2010; Leng et al., 2013; Puglisi et al., 2013). The current treatments such as chemotherapy and radiotherapy are likely having no effects on active cancer stem cells. However, the mechanism on how the cancer stem

cells are being regulated remains unknown. Based on the understanding of unique genetic properties of colon cancer stem cells, novel effective drugs should be designed to target on these cells for a better prognosis to successful treatment and cure (O'Brien et al., 2007).

Colon cancer cells may invade through the lymph vessels leading to lymph node metastases, or through the blood stream which result in widespread metastasis to distant organs within the abdominal cavity, such as the liver and lung (National Cancer Institute of the USA, 2013). Nevertheless, colon cancer is still one of the most potentially curable cancers amongst gastrointestinal cancers if it is detected not later than stage III cancer (Magaji et al., 2012), where it is not yet metastasised to distant sites. In general, patients survive for 5 years or more after diagnosis with treatment at an early stage. If the cancer does not recur within 5 years, the patient is considered cured (Chen, 2012).

Hence, screening is important as a routine prevention step for adults over the age of fifty. Besides, high-risk individuals require careful and routine follow-up to confirm no abnormal cell growth such as polyps and nonpolypoid lesions at the colon lining (Ackroyd and Hedberg, 1985). Screening procedures such as tumour marker carcinoembryonic antigen (CEA) blood test, faecal occult blood test (FOBT), sigmoidoscopy and colonoscopy are regularly used to detect colon cancer (Desch et al., 1999; Ouyang et al., 2005) when the

patient presents with symptoms such as abdominal discomfort, fatigue, loss of appetite, unexplained weight loss and changes in bowel movement: persistent diarrhoea or constipation, rectal bleeding, mucus or blood detected in stool (Neugut et al., 1993; Fijten et al., 1995; Minardi et al., 1998). However, the screening procedure stated above may have a chance to overlook small polyps, non-polypoid lesions and cancers. On the other hand, CEA is used in determining prognosis rather than a diagnosis test for colon cancer. Moreover, the CEA as a marker has low specificity as it is also elevated in breast and lung cancers and in other non-cancerous diseases. Hence, insufficient and slow development of improved early diagnosis and screening tools have resulted in most cancers escaping early detection, delaying timely treatment and leading to poor prognosis (Paranjape et al., 2009). Poor prognosis for patients with metastatic colon cancer and side effects of chemotherapy lead to high mortality rates in advanced-stage of this cancer.

Hereditary and environmental factors are important contributors to colon cancer development. According to National Cancer Institute of the USA, the risk of colon cancer increases with age, especially in individuals over age of 50 (Haggar and Boushey, 2009). Besides, individuals with a personal or family history of colon cancer or adenoma, polyps, nonpolypoid lesions and inflammatory bowel disease are also at higher risk of emerging colon cancer (Fernandez et al., 2004; Haggar and Boushey, 2009; Cummings and Cooper, 2011). The majority of colon cancer cases sporadically occur under the influence of environmental factors such as unhealthy dietary and cigarette

smoking habits (Haggar and Boushey, 2009). However, distinguishable genetic risk factors are not identified in most patients. Genetic variation such as single nucleotide polymorphisms (SNPs) may increase the sensitivity of cells towards environmental carcinogens (Aizat et al., 2013). Carcinogens such as polycyclic aromatic hydrocarbons (PAHs), heterocyclic amines (HCA) and N-nitrosamines that are present in cigarettes and processed food are caused damaging to the colon, either through direct consumption or the circulatory system, to induce DNA adducts in crypt cells and eventually cause mutations in the colon (Aizat et al., 2013). Acting together with the effects of environmental factors, environmentally sensitive genetic polymorphisms are known to influence cancer susceptibility (Christensen et al., 2009; Aizat et al., 2013).

Despite the multi-factorial aetiology of colon cancer, pathological changes of colon are predominantly caused by either the activation of cancer-associated protein-coding oncogenes and or the inactivation of tumour suppressor genes, respectively (Wu et al., 2011). These abnormalities cause alteration of expression of oncogenic or tumour suppressor proteins which in turn, affect the carcinogenesis processes, such as proliferation, apoptosis, metastasis, multidrug resistance and genetic and genomic stability maintenance (Wu et al., 2011). Hence, understanding the genetic and epigenetic alterations in different stages of progression of colon cancer provide valuable insight into the mechanism of tumorigenesis especially in metastatic colon cancer (Sarver et al., 2009; Kim et al., 2010).

2.1.1 Colon Cancer in Malaysia

Cancer statistics in Malaysia have been raised intensely in recent years. A total of 18,219 new cases were diagnosed in 2007 involving 8,123 males and 10,096 females (Ariffin and Saleha, 2011). Besides, cancer are likely more predominant among the Chinese than in the Malay and Indian ethnic groups (Ariffin and Saleha, 2011).

In Malaysia, colon cancer has now arisen to be the second most frequently diagnosed cancer in men and the third most frequent in women with most patients are above 50 years old (Ariffin and Saleha, 2011). Colon cancer has emerged as the third most common cancer-related death in both genders after lung and breast cancer, with a prevalence which is even higher than the worldwide cancer statistics (Aslam et al., 2009; Ariffin and Saleha, 2011).

Early diagnosis through blood or stool screening tests and colonoscopy is proven capable of significantly reducing mortality due to colon cancer and the occurrence of metastasis. However, colon cancer screening is not yet common in many countries (Ouyang et al., 2005). In Malaysia, screening guidelines recommend a yearly screening for individuals at average risk by using FOBT and colonoscopy (Yusoff et al., 2012). However, the acceptance of colon cancer screening is much lower than the more established cervical cancer screening probably due to anxiety towards invasive screening

approaches such as colonoscopy. Besides, Malaysian patients are mostly not aware of colon cancer risk factors and the importance of colon cancer screening (Yusoff et al., 2012). Therefore, the majority of colon cancers in Malaysia were first diagnosed at the late advanced-stage (Yusoff et al., 2012). Hence, colon cancer is chosen for this study in the hope to discover miRNA biomarkers for early diagnosis and better prognosis of this disease. Notably, the majority of previous studies have focused exclusively on either dysregulation of miRNA or alteration of genes associated with cancer phenotypes, but there is no attempt to conduct studies combining both aspects.

2.1.2 Colon Cancer Cell Lines

There are at least 44 reported cell lines derived from patients with colorectal adenocarcinoma in the American Type Culture Collection (ATCC). Among these cell lines, 23 are adherent type epithelial cells isolated from the colon, and four cell lines: HCT-15, SK-CO-1, HT-29 and WiDr were selected to serve as cell culture models for studying dysregulation of miRNA in colon cancer with miRNA expression profiling analysis in this dissertation. HCT-15 and SK-CO-1 were selected from the highly metastatic colorectal adenocarcinoma cell types; whereas HT-29 and WiDr were selected from the colorectal adenocarcinoma cell types with unknown cancer staging. These four cancer cell lines are widely used in various colon cancer-related researches.

According to ATCC, HCT-15 and SK-CO-1 cell lines are derived from male patients, and HT-29 and WiDr are cells derived from female patients; all four of them are biosafety level 1 cancer cell lines. These colon cancer cells grow as monolayer of clustered cells in appropriate culture media and adhered to the plastic surface of the culture flask. Both HT-29 and SK-CO-1 cells are positive for expression of oncogenes including c-myc, K-ras, H-ras, N-ras, myb, sis and fos. Furthermore, HT-29 cells are positive for the expression of secretory component of IgA, CEA and transforming growth factor beta binding protein and they do express urokinase receptors but do not have detectable plasminogen activator activity. WiDr cells express epidermal growth factor receptor (EGFR), CEA, colon specific antigen-p (CSAp), transforming growth factor beta (TGFβ), TP53 and positive for immunoperoxidase staining of keratin. HCT-15 cells are negative for CSAp but expressed CEA and positive immunoperoxidase staining of keratin.

2.2 microRNA (miRNA)

miRNA is a class of highly conserved single-stranded non-coding RNA molecules approximately 18 - 27 nucleotides (nt) in length (Lim et al., 2005; Choo, 2011). Since the first discovery of miRNA gene in 1993 (Lee et al., 1993), various studies have clearly shown their significant roles in post-transcriptional gene regulation by binding to the 3'-untranslated region (3'-UTR) of its target and further inducing targeted messenger RNA (mRNA) degradation or repressing protein translation (Lim et al., 2005; Guo et al., 2010;

Dong et al., 2011; Lai et al., 2012). miRNA expression is in turn, regulated by a number of general transcription factors, or proteins involved in carcinogenesis, such as c-Myc and TP53 (Slaby et al., 2009; Volinia et al., 2010; Ha, 2011; Yan et al., 2012). Thus, miRNAs are in the centre of a wide network of expression of genes critical to many important biological processes (Lu et al., 2008; Choo, 2011; Ha, 2011; Yan et al., 2012).

A single miRNA is revealed to regulate a few to hundreds of genes in various regulatory pathways (Calin and Croce, 2006; He et al., 2010) including cell cycle, cell death, MAPK signalling pathway, cancer progression and others. Therefore, miRNA-based therapies will have an advantage of one miRNA targeting the network of genes with minimal off-target side effects as compared to siRNA therapy (Ovcharenko, 2007). This is because siRNAs are pre-designed molecules that can target only single gene for disease treatment and these chemically synthesised oligonucleotide sequences might cause undesirable side effects to human body, while miRNAs are naturally expressed RNA molecules in human cells (Ovcharenko, 2007).

2.2.1 miRNA Biogenesis and Functions

The miRNAs biogenesis pathway involves multiple steps of processing of primary miRNA to mature miRNA species (Figure 2.1) (Winter et al., 2009). Briefly, miRNAs are derived from an endogenously expressed

transcript known as primary miRNAs. After the primary miRNA is cleaved by Drosha into a shorter miRNA-containing hairpin in the nucleus, it is now known as precursor miRNA, the precursor is further transported to the cytoplasm through specialised export machinery by Ran-GTP and Exportin-5 (Lee et al., 2002; Yi et al., 2003; Zeng and Cullen, 2003; Lund et al., 2004; Castanotto et al., 2009; Wang et al., 2011). The structure of the precursor miRNA is then cleaved by Dicer to create a 18-27 nt length miRNA:miRNA* duplex (Bartel, 2004; Castanotto et al., 2009; Tsutsumi et al., 2011). Upon dissociation of the duplex by helicase, the strand with the least thermodynamically stable 5' end is incorporated into a large multiprotein complex (Siomi and Siomi, 2009; Manavella et al., 2012), the RNA-induced silencing complex (RISC), which assembles to target mRNA via Watson-Crick base pairing between the guide strand and the complementary sequence at the target mRNA 3'-UTR to initiate post-transcriptional repression (Siomi and Siomi, 2009; Manavella et al., 2012). Target recognition is based on base pairing between the miRNA seed region (normally base 2 - 8 at the 5'end of miRNA) and its target. The degree of complementary sites between the guide and target determined the post-transcriptional gene silencing mechanism to take place (MacFarlane and Murphy, 2010). The target gene is silenced by mRNA cleavage if the miRNA seed sequence is perfect or near-perfect base pairing to the target (MacFarlane and Murphy, 2010). On the other hand, the target gene is silenced by translational repression or mRNA destabilization if the miRNA seed sequence is imperfectly base paired to the target transcript (MacFarlane and Murphy, 2010). Theoretically, both strands derived from the miRNA duplexes should be produced in equal concentration by Dicer

processing (Goedeke et al., 2013). However, accumulations of two miRNA arms are predominantly asymmetric at steady state (Guo and Lu, 2010; Yang et al., 2011). Hence, the most abundant strand of a processed precursor miRNA in cells is known as the guide strand, while the less abundant strand is known as the passenger strand or miRNA* (Guo and Lu, 2010).

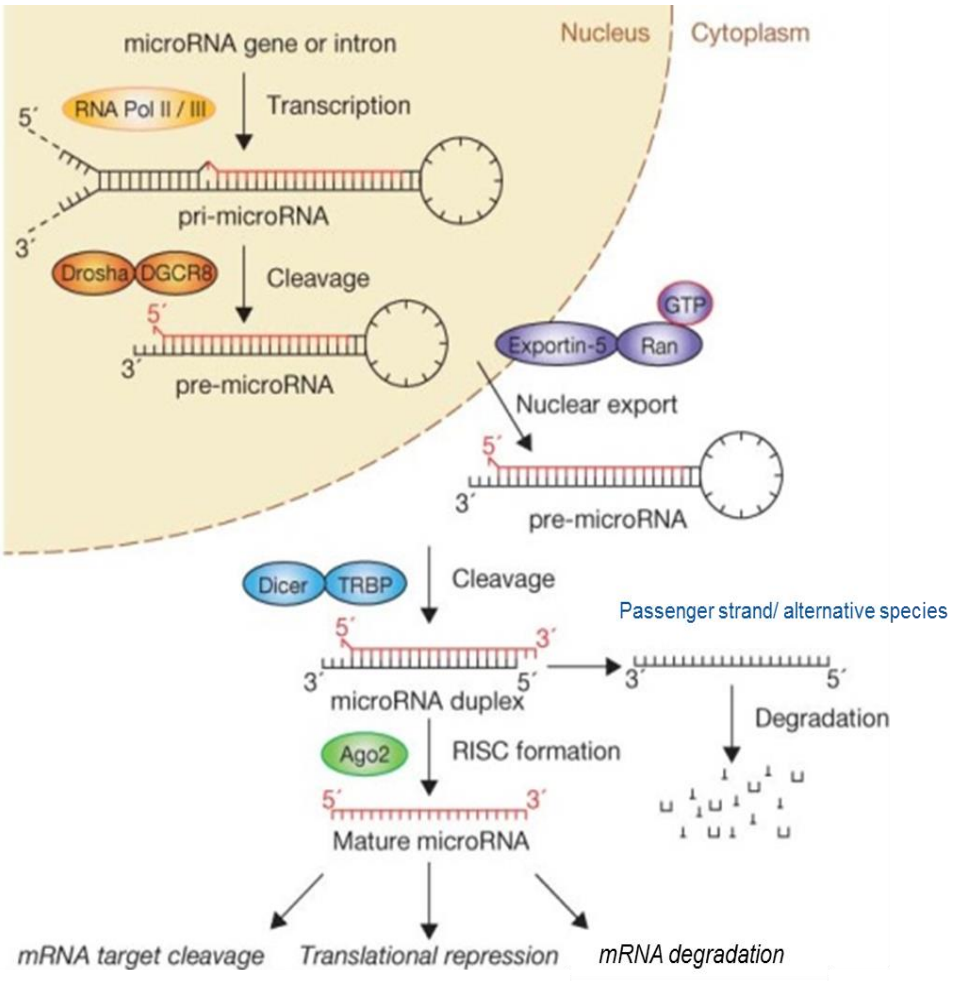


Figure 2.1: Biogenesis pathway of miRNA (Winter et al., 2009).

To date, according to miRBase release 20 (www.miRBase.org), a miRNA database, there are 1,872 miRNA precursors that have been reported producing probably over 2,578 mature miRNA species due to alternative-strand miRNA species generated by some precursors (Bartel, 2004; Siomi and Siomi, 2009). Most of the miRNAs are shown to be tissue-specific and some are cancer-specific. On the other hand, each miRNA may regulate a few to hundreds of distinct genes (Lim et al., 2005; Lai et al., 2012). Hence, a comprehensive analysis of involvement of miRNAs in dysregulated gene repression in cancers is a highly complex affair (Kusenda et al., 2006; Lu et al., 2008; Lundstrom, 2011; Sayed and Abdellatif, 2011; Cai et al., 2012). In addition, due to the small size of miRNAs, the lack of extensive base-pairing (approximately 6 - 8 bases in the seed sequence of a miRNA) between a miRNA and the targeted mRNA to achieve gene repression has also contributed to the complexity (Bartel, 2009). Although target prediction software could be helpful, understanding of cell type-specific expression patterns of miRNAs and their targeted genes are crucial in elucidating miRNA functions and contributions to carcinogenesis (Romero-Cordoba et al., 2012). Thus, screening a large pool of protein-coding genes involved in important biological processes, such as apoptosis, cell cycle and signalling, could also provide a more comprehensive picture of miRNA involvement in target genes leading to carcinogenesis (Slaby et al., 2009; Romero-Cordoba et al., 2012).

2.2.2 miRNA-5p and miRNA-3p: Guide and Passenger Strands

In most cases, the passenger strand, or the miRNA*(star) sequence is expressed in low levels or is undetectable (Figure 2.2) (Guo and Lu, 2010). Previous research has neglected the passenger miRNA strand due to its insignificant expression level in cells and is assumed to be not functional. However, recent researches have reported that some miRNA* sequences do act as functional guide miRNAs with abundant level of expression and are involved in various diseases, including cancers (Jazdzewski et al., 2009; Guo and Lu, 2010; Jiang et al., 2010; Jin et al., 2011; Kuchenbauer et al., 2011; López and Alvarez-Salas, 2011; Almeida et al., 2012). In the case where the miRNA* strands are reported as the abundant mature miRNA, the nomenclature miR-#-5p (5p means 5 primer or 5' from left arm) or miR-#-3p (3' from right arm) is denoted (Figure 2.2) (Guo and Lu, 2010). This is because both strands from the miRNA duplex have similar thermodynamic stability and energy at both termini of the miRNA duplex. Hence, there is no thermodynamic basis for the mature selection of either miRNA duplex ends (Mah et al., 2010). Both the mature sequences guide RISC to silence respective target mRNAs through mRNA cleavage, translational repression or mRNA degradation (Figure 2.2). Well-conserved miRNA* strands potentially offer opportunities in the target gene regulatory network. Guo and Lu (2010) reported about 80 species of human miRNA precursors that can yield the two alternatives (miR-#-5p and miR-#-3p) of abundant mature miRNAs with different seed sequences and target mRNAs, while most miRNA precursors

only yield abundant mature miRNAs from the left or the right arm, and the rare miRNA* sequences.

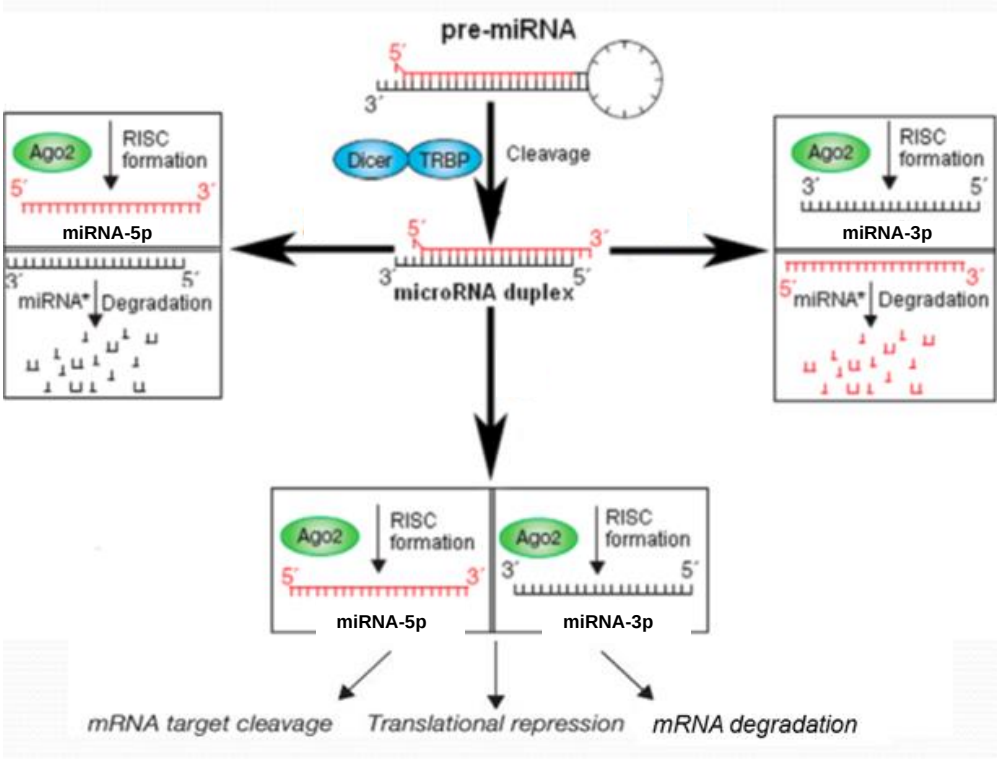


Figure 2.2: Mature selection of miRNA-5p and -3p (Guo and Lu, 2010).

miRNA* species have noticeable influence on vertebrate regulatory networks, as reported in Yang et al. (2011), and should be taken into account in studies of miRNA functions and their contribution to disease states. The microarray data provided transcriptome-wide evidence for the regulation of seed-matched targets for both miR-#-5p and -3p species of several miRNAs relevant to tumorigenesis, including miR-17, miR-34a, and miR-19 (Yang et al., 2011).

On the other hand, Eichner et al. (2010) reported that miR-378* expression is regulated by ERBB2 and induced a metabolic shift in breast cancer cells, from an oxidative to a glycolytic bioenergetics pathway, which is known as Warburg effect, a novel signature of cancer cell metabolism. miR-378* performs this function by inhibiting the expression of two PGC-1β partners, ERRγ and GABPA, leading to a reduction in tricarboxylic acid cycle (TCA cycle) gene expression and oxygen consumption as well as an increase in lactate production and in cell proliferation. Besides that, miR-378* expression was shown to be correlated with progression of human breast cancer (Eichner et al., 2010).

Furthermore, Jazdzewski et al. (2009) reported that polymorphic mature miRNAs from the passenger strand of pre-miR-146a (single nucleotide polymorphism of G and C base: miR-146a*G and miR-146a*C) contribute to

thyroid cancer. They also proposed that mature miRNAs from the passenger strand may regulate many genetic processes (Jazdzewski et al., 2009).

There are growing evidences suggest that mature sequences derived from both ends of the miRNA precursor may be biologically functional. Therefore, in 2013, miRBase started to substitute the original nomenclature of mature sequence miRNA and miRNA*(star), the guide strand and passenger strand, with new nomenclature miR-#-5p and -3p for sequences derived from the 5' and 3' end of the miRNA precursor (Kozomara and Griffiths-Jones, 2014), regardless of their expression levels in cells. Hence, the nomenclature miRNA*(star) is still commonly used in miRNA research before the year of 2014.

In this study, the dysregulation of miRNAs derived from 5p and 3p and their respective downstream target transcripts were focused.

2.3 miRNAs in Cancers

As a consequence of the vital gene regulatory roles of miRNAs in numerous metabolic and cellular pathways controlling cell proliferation, differentiation and survival, it is not surprising that alterations in the miRNA expression contribute to the pathogenesis of various human diseases (Lu et al.,

2008; Aslam et al., 2009; Jazdzewski et al., 2009; Ryan et al., 2010; Ha, 2011; Lundstrom, 2011; Zhu et al., 2012). There are also increasing evidences demonstrating a relationship of miRNAs to various types of cancers as dysregulation of miRNA expression profiles has been revealed in most tumours examined (Calin et al., 2002; Ha, 2011; Cai et al., 2012).

Yan et al. (2012) demonstrated that transcription factor (TF)-miRNA feed-forward loops may be common drivers of the development of various cancers. The regulation mechanisms between TF, miRNA and the common target genes form the miRNA-mediated feed-forward loops (FFL). The miRNA common target genes are also known as FFL target genes, as TF regulates transcription of the miRNA and both TF and miRNA regulate a similar set of targets (Yan et al., 2012). These FFL networks are recurrent and govern many normal cell functions and are functional in the larger regulatory network. For instance, the FFL consisting of c-Myc, the miR-17 cluster and *E2F1* modulates cellular proliferation in cancer (Yan et al., 2012). Thus, altered regulatory network is able to be identified by combining the data sets of miRNA, TF and target genes expressed in normal and cancer, hence, understanding of the causes of cancer and discovery of novel drug targets are possible.

2.3.1 miRNAs as Tumour Suppressors or Oncogenes in Cancer

Lu et al. (2008) have reported that miRNAs often emerge as defined miRNA groups for a particular state of pathology such as cancers. These unique sets of miRNAs might be used for development of assays for diagnosis and prognosis. In cancers, miRNAs could function as either or both as oncogenes or tumour suppressor genes depending on the genes being regulated (Zhang and Chen, 2009; Ha, 2011; Romero-Cordoba et al., 2012; Yan et al., 2012). Besides, the enzyme factors or genes that are required for the biogenesis of miRNAs have been associated with various cancers and might themselves function as oncogenes and tumour suppressors (Esquela-Kerscher and Slack, 2006). However, due to different miRNA expression patterns for specific tissue and cancer differentiation states, it is difficult to classify them as oncogenes or tumour suppressor genes (MacFarlane and Murphy, 2010). It still remains unclear whether alterations of miRNAs are the direct or indirect cause of the cancer.

Oncogenic miRNAs are frequently up-regulated in cancer to promote cancer-related phenotypes and to sustain growth of cancer cells (Zhang et al., 2007; Shenouda and Alahari, 2009). As shown in Table 2.1 (Garzon et al., 2009), the miR-17-92 cluster and miR-21 are up-regulated in acute myelogenous leukaemia, glioblastoma, colon cancer, stomach cancer and breast cancer. These miRNAs are located particularly in the minimal regions of amplification (Zhang et al., 2007; Shenouda and Alahari, 2009). Tumour

suppressive miRNAs are often found to be down-regulated in cancer (Zhang et al., 2007; Shenouda and Alahari, 2009). For example, members of the miR-29 family are down-regulated in lung cancer, chronic lymphocytic leukaemia (CLL) and acute myelogenous leukaemia (AML) (Table 2.1). Members of the miR-29 family are revealed to function as tumour suppressive miRNAs to induce apoptosis and reduce tumorigenecity (Table 2.1). However, their functions in repressing malignant transformation of normal cells are lost due to genetic variations including genomic deletions, mutations, epigenetic silencing and miRNA processing alterations (Garzon et al., 2009). These miRNAs are often localised at the cancer-related genomic regions – the minimal regions of loss of heterozygosity (LOH) (Zhang et al., 2007; Shenouda and Alahari, 2009).

Table 2.1: miRNA with experimental data supporting tumour suppressor or oncogene function in cancer (Garzon et al., 2009)

miRNA	Expression in patients	Confirmed targets	Experimental data	Function
miR-15a miR-16-1	Down-regulated in CLL[1]	Bcl-2, Wt-1	Induce apoptosis and decrease tumorigenicity	TS[1]
let-7 (a,-b,-c,-d)	Down-regulated in lung and breast cancer	RAS, c-myc, HMGA2	Induce apoptosis	TS
miR-29 (a,-b,-c)	Down-regulated in CLL, AML[1](11q23), lung and breast cancers, and cholangiocarcinoma	TCL-1, MCL1, DNMT3s	Induce apoptosis and decrease tumorigenicity	TS
miR-34a-b-c	Down-regulated in pancreatic, colon, and breast cancers	CDK4, CDK6, cyclinE2, E2F3	Induce apoptosis	TS
miR-155	Up-regulated in CLL, DLBCL[1], FLT3-ITD[1], AML, BL[1], and lung and breast cancers	c-maf	Induce lymphoproliferation, pre-B lymphoma/ leukemia in mice	OG[1]
miR-17~92 cluster	Up-regulated in lymphomas and in breast, lung, colon, stomach, and pancreas cancers	E2F1, Bim, PTEN	Cooperates with c-myc to induce lymphoma in mice, transgenic miR-17-92 develop lymphoproliferative disorder	OG
miR-21	Up-regulated in breast, colon, pancreas, lung, prostate, liver, and stomach cancer; AML(11q23); CLL; and glioblastoma	PTEN, PDCD4, TPM1	Induce apoptosis and decreases tumorigenicity	OG
miR-372/ miR-373	Up-regulated in testicular tumours	LATS2	Promote tumorigenesis in cooperation with RAS	OG

[1]Abbreviations: CLL, chronic lymphocytic leukemia; AML, acute myeloid leukemia; DLBCL, diffuse large B cell lymphoma; FLT3-ITD, FMS-like tyrosine kinase 3 in tandem duplication mutations; BL, Burkitt's lymphoma; TS, tumour suppressor; OG, oncogene.

2.3.2 miRNAs in Colon Cancer

A range of dysregulated miRNAs was previously identified to be associated with CRC pathogenesis, progression and therapeutic responses (Luo et al., 2011). The CRC pathway derived from KEGG Pathway Database is shown in Figure 2.3. Colon cancer is arisen in a step-wise manner from the colon epithelium to become benign adenoma (polyp or polypoid lesion), predominantly due to accumulation of genetic variations or alterations of the oncogenes and tumours suppressor genes (Wu et al., 2011). The polypoid lesion is then progressively developed to invasive adenocarcinoma and later on distant metastasis (Wu et al., 2011). According to KEGG pathway database, there are two main mechanisms of genomic instability identified in CRC tumorigenesis: chromosomal instability and microsatellite instability pathways (Figure 2.3). Chromosomal instability arises from a series of genetic alterations involving the activation of oncogenes such as β-catenin and *KRAS* and also the inactivation of tumour suppressor genes such as *APC*, *DCC*, *SMAD2*, *SMAD4* and *TP53* (Kanehisa and Goto, 2000; Kanehisa et al., 2014). The second pathway, the microsatellite instability pathway, is a result of inactivation of DNA repair genes *MLH1*, *MSH2*, *MSH3* and *MSH6* by promoter hypermethylation and also secondary mutation of *TGFBR2* and *BAX* genes (Kanehisa and Goto, 2000; Kanehisa et al., 2014). Besides the major chromosomal instability and microsatellite instability pathways, there are a number of other pathways involved in tumorigenesis process of CRC, including apoptosis, cell cycle, p53 signalling pathway, PI3K-Akt signalling pathway and Wnt signalling pathway (Figure 2.3) .

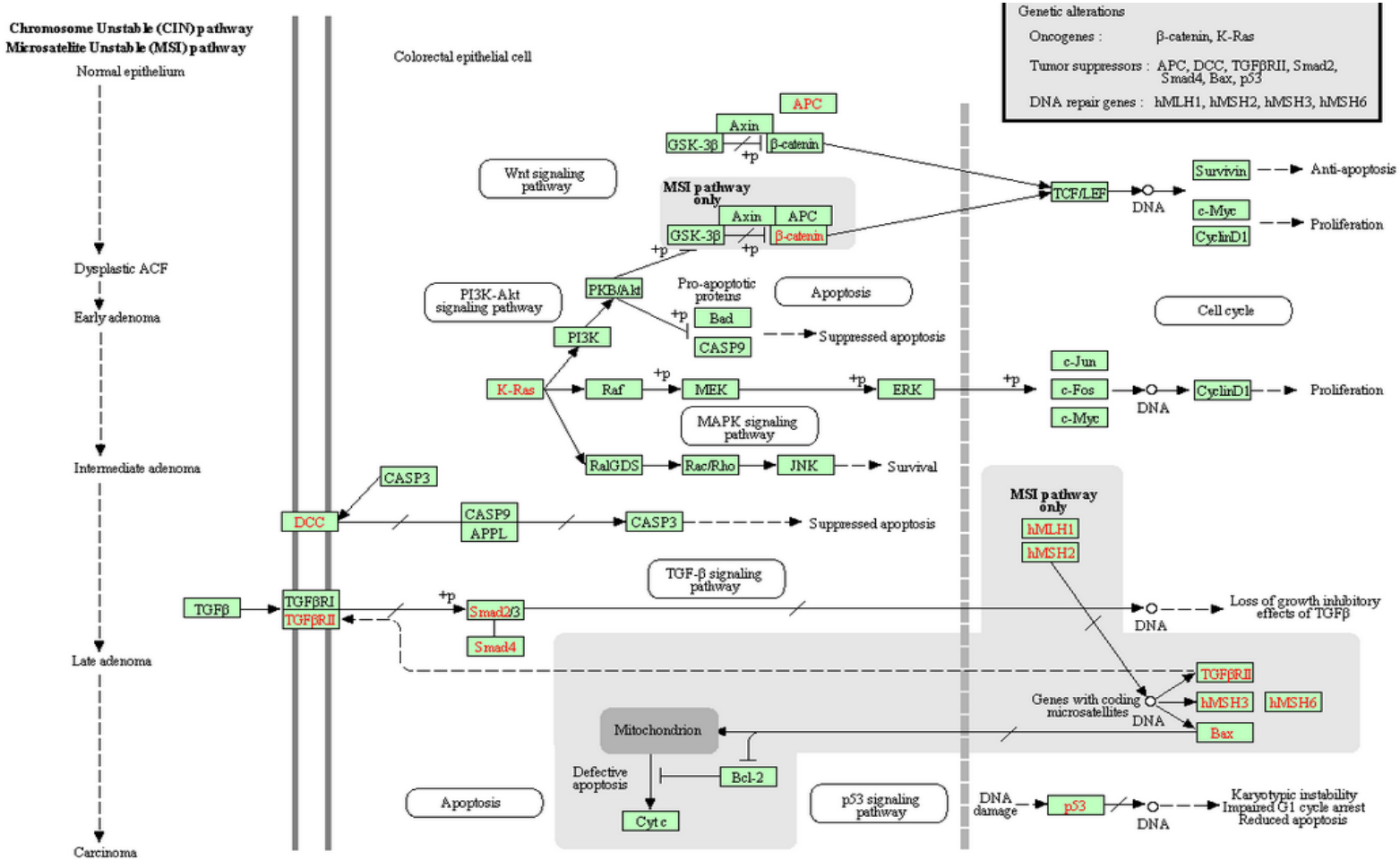


Figure 2.3: Colorectal cancer pathway. Adapted from KEGG pathway database (Kanehisa and Goto, 2000; Kanehisa et al., 2014).

2.3.3 miRNA Associated with Cancer-related Phenotypes and Pathways

Uncontrolled cell proliferation is commonly revealed in malignancy (Wu et al., 2011). Activated Ras/Raf/mitogen activated protein kinase extracellular signal-regulated kinase (MAPK-ERK) cascade is reported promoting cell proliferation in colon cancer (Calcagno et al., 2008; Wu et al., 2011). A number of miRNAs including let-7a (Akao et al., 2006), miR-143 (Chen et al., 2009) and miR-18a* (Tsang and Kwok, 2009), were reported to be involved in inhibiting cell proliferation in colon cancer via targeting on KRAS protein. miR-143 is regularly down-regulated in colon cancer and significantly suppresses CRC cell growth through inhibition of KRAS translation (Slaby et al., 2007; Ng et al., 2009; Akao et al., 2010). Apart from KRAS, Wu et al. (2011) and others revealed that extracellular signal-regulated kinase 5 (ERK5) and DNA methyltransferase 3A (DMNT3A) were direct targets of miR-143 (Borralho et al., 2009; Ng et al., 2009). The tumour-suppressive role of miR-143 in the epigenetic aberration of CRC provides a potential development of miRNA-based targeted approaches for CRC therapy. In contrast, the up-regulation of miR-200b by chemotherapeutic drug 5-fluorouracil, represses the KRAS-inhibiting PTPN12, which in turn, indirectly activated KRAS. This suggests that miR-200b-mediated KRAS activation could serve as an auto-regulatory mechanism to neutralise the cytotoxic effects of 5-fluorouracil (Rossi et al., 2007; Wu et al., 2011).

The dysregulation of miRNA has been shown to involve in apoptosis regulatory pathway by affecting anti-apoptotic Bcl-2 family members' expressions in colon cancer (Wu et al., 2011). For example, the down-regulation of miR-195 is shown to promote apoptosis in colon cancer cells HT-29 and LoVo by targeting the Bcl-2 protein (Liu et al., 2010).

Furthermore, a few miRNAs have been shown to involve in the regulation of colon cancer cells invasion and metastatic processes (Wu et al., 2011). For example, miR-196a promotes the detachment, migration and invasion of colon cancer cells by direct targeting the homeobox genes, a class of transcription factors (Schimanski et al., 2009). On the other hand, the up-regulation of miR-21 had been demonstrated to target tumour suppressor programmed cell death protein 4 (PDCD4), which is frequently down-regulated in solid tumours and is associated metastasis of colon cancer cells (Yamamichi et al., 2009), whereas the up-regulation of miR-141 inhibits mesenchymal-to-epithelial transition (MET) by targeting SIP1, an E-cadherin transcriptional repressor (Hu et al., 2010; Wu et al., 2011). Other miRNAs such as miR-26, miR-145 and miR-200c are also involved in the control of invasiveness and metastasis in CRC (Burk et al., 2008; Arndt et al., 2009; Ma et al., 2011; Wu et al., 2011).

Besides, miRNAs have been reported in regulating other cancer-related phenotypes. For instance, miR-107 inhibits angiogenesis, the new blood vessel

formation process, through suppressing hypoxia-inducible factor β (HIFβ)-mediated expression of vascular endothelial growth factor (VEGF) (Yamakuchi et al., 2010; Wu et al., 2011). Furthermore, miRNA is found to be involved directly in the genetic integrity maintenance. For example, miR-155 promotes mutation by targeting of three enzymes: MSH2, MSH6, MLH1, which are involved in DNA repair (Haggar and Boushey, 2009; Valeri et al., 2010; Wu et al., 2011). Apart from that, miR-200 family, which is activated by ZEB1, inhibits stem cell-like properties in colon cancer cells via targeting on SOX2 and KLF2 (Wellner et al., 2009). Besides, miR-21 and miR-181b-1 were revealed to associate inflammation to cancer, target phosphatase and tensin homolog and CYLD, respectively, to enhance nuclear factor-kappa B (NFκB) activity (Iliopoulos et al., 2010). The transcriptional signatures of the above-mentioned miRNAs could be detected mainly in primary colon cancer tissues (Iliopoulos et al., 2010; Wu et al., 2011).

2.4 Potential Applications of miRNA for Cancer Management in Clinical Settings

miRNA expression profiles are better suited for cancer biomarkers discovery as compared to the existing cancer biomarker CEA or cancer-associated gene expression profiles. Previous studies showed that miRNAs are better in classifying human cancers of unknown primary origin and poorly differentiated tumours better than mRNA classifier (Lu et al., 2005). Furthermore, most of the miRNAs are tissue-specific and some are cancer-

specific (Calin and Croce, 2006), e.g. miR-143 and miR-145 are specifically down-regulated in colon cancer (Michael et al., 2003; Pekow et al., 2012; Takaoka et al., 2012).

miRNA profiling in sera and plasma samples can serve as a minimal invasive approach to discover important new biomarkers for a wide range of critical diseases and biological processes (Ha, 2011). Recent reports demonstrated that miRNAs isolated from blood may suitable for early cancer diagnosis markers. Wu et al. (2011) revealed that both normal and cancer cells released miRNAs into peripheral blood and circulated in a highly-stable complex, known as exosomes or microvesicles, which was protected from RNase degradation (Hunter et al., 2008; Kosaka et al., 2010), and the miRNA levels were reproducible among individuals (Grady and Tewari, 2010; Shen et al., 2011; Wu et al., 2011; Weiland et al., 2012). Besides, plasma and serum miRNAs were found to be stable and robust even after few cycles of freezing and thawing (Ha, 2011).

Previous reports investigated the expression levels of 95 miRNAs in plasma of CRC patients, and revealed that miR-17-3p and miR-92a were significantly increased in plasma (Ng et al., 2009; Wu et al., 2011). Surprisingly, the plasma levels of these two miRNAs were significantly reduced following curative surgery to remove tumour tissues. Moreover, miR-92a is able to differentiate CRC from gastric cancer, inflammatory bowel

diseases (IBD) and healthy subjects. Another report shown that miR-92a and miR-29a were able to differentiate healthy subjects from patients with CRC and advanced adenomas, suggesting that these miRNAs could be useful for early diagnosis and monitoring of CRC (Huang et al., 2010; Wu et al., 2011).

The combined application of miRNA-based gene therapy and chemotherapy is possibly an effective or could be the best available approach to tackle tumorigenesis processes (Li et al., 2009). Altering specific miRNA levels in cancer cells using miRNA replacement or inhibitor technologies can restore regular miRNA activities and repair the gene regulatory network and signalling pathways, which in turn, reverse the cancerous phenotypes of cells (Yu et al., 2013). A proposed scheme for the treatment of liver cancer with combined chemotherapy and miRNA-based therapy is shown in Figure 2.4 (Lujambio and Lowe, 2012).

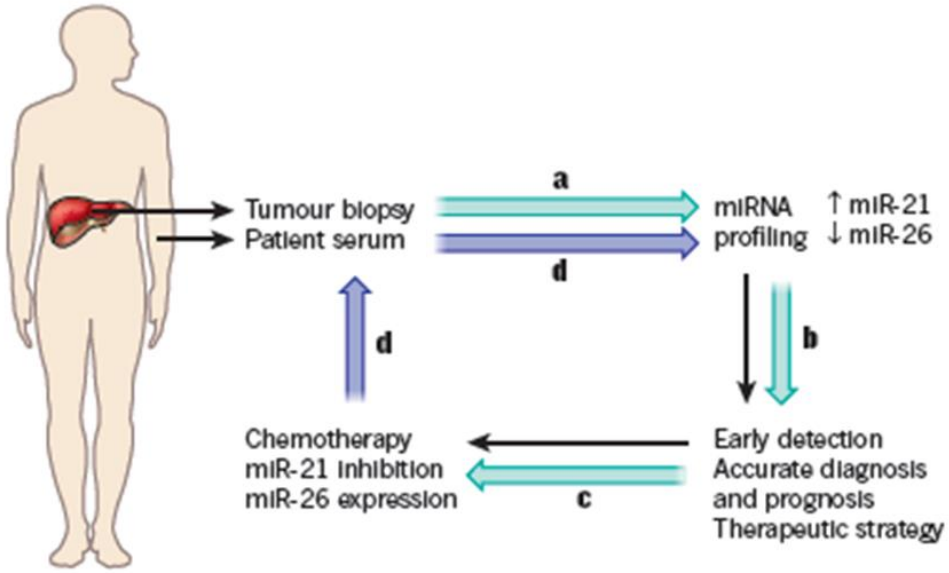


Figure 2.4: A proposed scheme for the treatment of liver cancer with combined chemotherapy and miRNA-based therapy (Lujambio and Lowe, 2012).

Briefly, liver tumour biopsies and patient blood serum were first extracted and further subjected to miRNA expression profile by using miRNA microarray analysis. miRNA diagnostic markers, as showed in Figure 2.4 (Lujambio and Lowe, 2012), miR-21 (oncogenic miRNA) and miR-26 (tumour suppressive miRNAs) were identified in the clinical specimens. The identified oncogenic miRNAs in miRNA profiling of tumour and serum could be inhibited by using different strategies, for example locked nucleic acid constructs or specific miRNA inhibitor for miR-21. On the other hand, down-regulation of tumour-suppressive miRNA in the tumour could be restored with miRNA replacement or specific miRNA mimic for miR-26. After treatment, the patient could be monitored for recurrence of cancer periodically by investigating miRNA levels in circulating blood, which is a minimal invasive approach, compared to tumour biopsies extraction. If miR-21 was detected in blood serum during the monitoring screening, this indicates a potential relapse, and treatment would resume. Therefore, early diagnosis for cancer is possible by screening cancer with miRNA markers using a less invasive approach, which then leads to a better prognosis and miRNA-based therapeutic strategies.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Culture

Cell culture was performed in an Airstream Class II Biological Safety Cabinet (ESCO, Singapore) under aseptic conditions. A 37 °C cell culture incubator (ESCO, Singapore) supplied with 5% CO_2 was used for maintenance of colon cancer cells. An inverted phase contrast light microscope (Nikon, Japan) was used to examine the colon cancer cells. Disposable cell culture T-flask sizes of 25 cm^2 and 75 cm^2 with filter cap (Thermo Fisher Scientific, USA), culture dishes and multiwell plates (TPP, Switzerland), serological pipette sizes of 5 mL and 10 mL (Omnicell, Singapore), and conical centrifuge tubes of 15 mL (Nest Biotechnology, China) were used for cell culture work. Media bottles and pipette tips were sterilised in an autoclave (HVE-50, Hirayama, Japan) before use in culture work.

3.1.1 Colon Cancer Cell Lines

In this work, four human colorectal adenocarcinoma cell lines, HCT-15, HT-29, SK-CO-1 and WiDr, were used. HCT-15, SK-CO-1 and WiDr cells were obtained from American Type Culture Collection (ATCC,

Manassas, VA, USA). HT-29 cells were kindly provided by Associate Professor Dr. Noorjahan Banu Mohamed Alitheen, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia.

3.1.2 Preparation of Culture Medium

Culture media were prepared according to the manufacturer's guidelines. Dulbecco's Modified Eagle Medium (DMEM) powder (GIBCO, USA) was dissolved together with 3.7 g of sodium bicarbonate (Merck KGaA, Germany) in 900 mL of ddH_2O. The pH of the medium was adjusted to pH 7.2 and the volume was topped up to one litre. The medium was filter sterilised with a 0.2 µm cellulose acetate membrane filter unit (TPP, Switzerland) using a vacuum pump system, stored in a media bottle and kept at 4 °C. Minimum Essential Medium (MEM) (GIBCO, USA) was prepared using the same procedure as for DMEM medium preparation, with further addition of 2.2 g sodium bicarbonate. To make a complete culture medium, 10% (v/v) foetal bovine serum (FBS) (GIBCO, USA), 2 mM L-glutamine, and 1% antibiotic-antimycotic (100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone, GIBCO, USA) were added to the filter-sterilised medium.

3.1.3 Revival of Cells from Liquid Nitrogen Frozen Stock

A cryovial of frozen cells was removed from liquid nitrogen storage and was rapidly thawed by gently swirling in a 37 °C water bath until few ice crystals were observed. The vial was sterilised with 70% ethanol before the cap was opened. The thawed cells were transferred drop-wise into 9 mL pre-warmed complete culture medium and the cells were gently mixed. Cryoprotectant agent dimethyl sulfoxide (DMSO) was removed by centrifugation at 1,800 rpm for 5 min in a benchtop centrifuge. The cell pellet was then gently resuspended in 1 mL of fresh pre-warmed complete medium and the cell suspension was transferred into a 25 cm^2 T-flask containing 5 mL complete medium. The culture was maintained in a 37 °C cell culture incubator supplied with 5% CO_2 under humidified atmosphere.

3.1.4 Maintenance of Cell Culture and Subculturing

HCT-15 and HT-29 cells were maintained in DMEM medium while SK-CO-1 and WiDr cells were maintained in MEM medium. Culture medium was replaced twice a week. For subculturing, spent culture medium was discarded and the cells were washed twice with 1X PBS, pH 7.4 (MP Biomedicals, USA) and the flask was gently rocked back and forth several times and the wash solution was removed. One mL of pre-warmed 0.25% trypsin-EDTA solution (GIBCO, USA) was added to the flask and placed at 37 °C for 3 min to facilitate cell detachment. One mL of serum-containing

culture medium was added to the detached cells to inactivate trypsin. The cell suspension was transferred to a 15 mL conical tube and was pelleted by centrifugation at 1,800 rpm for 5 min. The cell pellet was resuspended in 1 mL pre-warmed complete culture medium. Total cell number was counted using a hemacytometer and 0.4% (w/v) trypan blue solution in PBS (MP Biomedicals, USA). Cell concentration was determined by the formula as showed in the next page.

Cell concentration

Cell number per mL = (the average counted cells) x (dilution factor) x (10^4)

The cell suspension was then diluted and seeded at ratio of 1:3 to new flasks.

3.1.5 Cryopreservation of Cultured Cells

Cells were detached following the procedure used for subculturing. Total cell number and cell viability were determined using a hemacytometer and healthy cells with a viability rate > 90% were cryopreserved. Cells at a density of 5 x 10^6 cells per mL were pelleted by centrifugation at 1,800 rpm for 5 min. The pellet was gently resuspended in 1 mL cold freezing medium containing 90% (v/v) FBS and 10% (v/v) DMSO. Aliquot of the cell suspension was dispensed into cryovial (Corning, USA) and placed in a styrofoam storage rack. The cells were first kept in a -20 °C freezer for 4 h before being transferred to a -80 °C deep freezer overnight followed by transferring to liquid nitrogen for long-term storage.

3.2 RNA Preparation

3.2.1 RNA Isolation

Total RNA was isolated from colon cancer cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's guidelines. The RNA isolation procedures were carried out at room temperature. Approximately 5 x 10^6 cells were trypsinised and cell pellet was collected by centrifugation at 1,800 rpm for 5 min and the cells were lysed in 600 µL Buffer RLT Plus. The lysate was then transferred to a QIAshredder spin column placed in a 2 mL collection tube (Qiagen, USA) and the lysate was homogenised by centrifugation at full speed of 14,800 rpm for 2 min. The homogenate was transferred to a gDNA Eliminator spin column and subsequently genomic DNA in the lysate was removed by centrifugation at 12,000 rpm for 30 s. Then, 600 µL 70% ethanol was added to the flow-through which was then mixed well by pipetting. The lysate was transferred to an RNeasy spin column. The spin-column was spun down at 12,000 rpm for 30 s and the flow-through was discarded. The membrane of spin-column was washed once with Buffer RW1 and twice with Buffer RPE by centrifugation at 12,000 rpm for 30 s and the flow-through was discarded. RNA was finally eluted with 30 µL non-DEPC treated RNase-free water by centrifugation at 12,000 rpm for 1 min. RNA was stored at -80 °C for future use.

3.2.2 RNA Quantification and Integrity Assessment

The concentration of isolated RNAs was determined by measuring absorbance value at 260 nm (A_{260}) in a NanoPhotometer (Implen, Munich, Germany). Purity of RNA was estimated by the ratio of the absorbance 260 nm and 280 nm readings (A_{260}/A_{280}). Only RNAs with a purity achieving A_{260}/A_{280} ratio of 1.8 – 2.1 were qualified for miRNA profiling analysis and subsequent profiling data validation by real-time quantitative reverse transcription PCR (PCR).

Integrity of the isolated RNA was also examined by agarose gel electrophoresis. One µL isolated RNAs were mixed with 1 µL 6X gel loading dye (Thermo Fisher Scientific, USA) and 4 µL H_2O and were subjected to 1% agarose gel electrophoresis at 80 V for 45 min using 1X TBE running buffer (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA, pH 8.0). The gel was stained with 3X GelRed nucleic acid stain (Biotium, USA) and visualised by exposure to 302-nm UV light under BioSpectrum Imaging System (UVP, USA). RNAs with sharp bands of 28S and 18S ribosomal RNA (rRNA) with an approximately 2:1 ratio were considered intact.

3.3 Global miRNA Expression Profiling Analysis

3.3.1 miRNA Real-Time PCR Array Analysis

Good-quality RNA samples of the four colon cancer cell lines were sent for global miRNA expression profiling service provided by WaferGen Biosystems. Two commercially purchased RNA samples isolated from two individual sources of non-cancerous colonic tissues (Origene, USA) were included in the study as normal. RNA quality was confirmed again using NanoDrop ND-1000 (Thermo Fisher Scientific, USA) and 2100 Bioanalyzer (Agilent Technologies, CA, USA) by WaferGen. One µg total RNA was analysed using the SmartChip Human MicroRNA Panel V2 (WaferGen Biosystems, Fremont, CA, USA), a nanoscale miRNA real-time PCR array containing 1,190 miRNA-specific reactions in quadruplicates for a total of 5,184 reactions per sample chip. miRNA was first ligated to a pre-adenylated linker (3' adapter) by RNA ligase 2 at 22 °C for 60 min. Subsequently, the ligated RNA was subjected to one-step on-chip real-time PCR reaction at 52 °C for 5 min, 95 °C for 10 min, 95 °C for 1 min and 52 °C for 1 min, followed by 39 amplification cycles of denaturation at 95 °C for 1 min and annealing at 60 °C for 1 min to synthesise cDNA and to amplify target on the SmartChip Cycler (WaferGen Biosystems, USA). Additional nineteen endogenous and four exogenous controls were included for data quality control in this analysis.

3.3.2 Profiling Data Analyses

The raw data obtained from the miRNA profiling analysis were cycle threshold (C_T) values. C_T is defined as the fractional number of PCR cycles required for the fluorescent signal to cross the threshold of detection in real-time PCR. In the SmartChip Human MicroRNA panel analysis, the cut-off value for C_T was set at 30 as designated by the service provider. Data were analysed using the comparative cycle threshold ($\Delta\Delta C_T$) method to obtain the relative $\log_2$(fold change) of miRNAs, in which the C_T value of each sample for each specific miRNA was normalised to that of the endogenous controls. Then, the colon cancer cell samples were again normalised to those of the non-cancerous colonic tissues to quantify the relative expression level of each specific miRNA in colon cancer cells.

Further analysis was performed to extract relevant information from the complex miRNA expression profiles in the colon cancer cell samples. The C_T values obtained from the miRNA expression profiling analysis was first analysed using the WaferGen in-house macro-enabled excel template. The C_T values were further applied to SABiosciences miScript miRNA PCR Array Data Analysis Web Portal (Qiagen, USA) for statistical analysis, scatter plot, volcano plot and hierarchical clustering analysis. Differentially expressed miRNAs that showed > 1.5 fold differences, whether they are up- or down-regulated in colon cancer cells relative to the non-cancerous colonic tissues, were identified for further validation.

3.3.3 Nomenclature

Throughout this work, the miR-#-5p and -3p nomenclature as recommended by miRBase was used to substitute the original mature sequence miRNA and miRNA*(star) species (Kozomara and Griffiths-Jones, 2014). The original miRNA names are still available in the database for cross referencing. All miRNAs described in this work are human (hsa)-miRNA. For simplicity, the hsa- prefix has been dropped from all miRNA designations in this dissertation.

3.4 Primers for Real-Time PCR: miRNA and mRNA Quantification

For miRNA real-time PCR, mature sequences of selected miRNAs were extracted from the miRBase database (www.miRBase.org) and synthesised as specific forward primers to amplify the selected miRNAs. The Universal qPCR Primer (Invitrogen, CA, USA) was used as reverse primer in the miRNA assay.

For real-time PCR on mRNA quantification, primers for *IGF1R* (Lim et al., 2011) and *GAPDH* (Zhang et al., 2011a) were checked using Basic Local Alignment Search Tool (BLAST) and global alignment algorithm to ensure the primer pair sequences are specific and do not match other undesirable gene sequence published in the National Center for Biotechnology

Information (NCBI) database in order to avoid amplification of non-specific sequences in the assay. Primer pair for *KRAS* gene was designed using NCBI Primer-BLAST (Ye et al., 2012). All primers were synthesised by 1st BASE Oligos (Singapore) and are listed in Tables 3.1 and 3.2.

Table 3.1: Primers used in miRNA real-time PCR

miRNA	Accession	Forward primer sequence (5' – 3')
miR-20a-5p	MIMAT0000075	TAAAGTGCTTATAGTGCAGGTAG
miR-20a-3p	MIMAT0004493	ACTGCATTATGAGCACTTAAAG
miR-378a-5p	MIMAT0000731	CTCCTGACTCCAGGTCCTGTGT
miR-378a-3p	MIMAT0000732	ACTGGACTTGGAGTCAGAAGG
let-7d-5p	MIMAT0000065	AGAGGTAGTAGGTTGCATAGTT
let-7d-3p	MIMAT0004484	CTATACGACCTGCTGCCTTTCT
RNU6	X07425	CACCACGTTTATACGCCGGTG

Table 3.2: Primers used in real-time PCR for mRNA quantification

Gene	Accession	Primer sequence (5' – 3')	Amplicon size (bp)
KRAS	NM_004985	F: TGAGGACTGGGGAGGGCTTTCTT R: AGAAGGCATCATCAACACCCTGTCT	255
IGF1R	NM_000875	F: CAAGGCCTGAAAACTCCATC R: CGCTGATCCTCAACTTGTGA	106
GAPDH	NM_001256799	F: GAAATCCCATCACCATCTTCCAGG R: GAGCCCCAGCCTTCTCCATG	120

F: Forward primer sequence; R: Reverse primer sequence

3.5 Validation of Profiling Data by miRNA Real-Time PCR

Expression profiles of three selected pairs of miRNAs (see Table 3.1) were confirmed by standard miRNA real-time PCR using the NCode SYBR GreenER miRNA qRT-PCR Kit (Invitrogen, USA) in a Rotor-Gene Q real-time PCR cycler (Qiagen, USA). The miRNA assay was performed according to workflow shown in Figure 3.1. Briefly, miRNA species in 1 µg total RNA was polyadenylated in a 25 µL reaction volume containing poly A polymerase and 80 µM ATP in 1X miRNA Reaction Buffer with 2.5 mM $MnCl_2$, incubated in a heat block at 37 ˚C for 15 min. To synthesise first-strand cDNA, reverse transcription of the tailed miRNA population was performed. Four µL poly(A)-tailed RNA was incubated at 65 ˚C for 5 min with Universal RT Primer and Annealing Buffer. The reaction was then added to a final volume of 20 µL with 1X First-Strand Reaction Mix and SuperScript III Reverse Transcriptase (RT)/RNaseOUT Enzyme Mix provided by the kit. The reaction tube was briefly spun down and incubated in a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) at 50 °C for 50 min and 85 °C for 5 min to inactivate the reaction.

Synthesised cDNA was subjected to real-time PCR (Table 3.3) using SYBR GreenER qPCR SuperMix Universal reagent (Invitrogen, USA) containing mixture of hot-start *Taq* DNA polymerase, SYBR GreenER fluorescent dye, UDG, dNTPs, $MgCl_2$; the specific miRNA sequence was then amplified using a miRNA-specific forward primer designed (Table 3.1) and

Universal qPCR primer (Invitrogen, USA) as the reverse primer. Negative control was included in the assay by substituting cDNA with DEPC-treated water to ensure no contaminant was detected in the PCR reactions. The reactions were incubated in a Rotor Gene Q cycler (Qiagen, USA) for UDG incubation at 50 °C for 2 min, UDG inactivation and hot-start DNA polymerase activation at 95 °C for 10 min, followed by 40 amplification cycles of 15 seconds denaturing interval at 95 °C and 60 seconds primer annealing step at 58 °C. The quality of the cycling products was validated in a melt-curve analysis by slowly heating the PCR products from 58 °C to 95 °C in 0.3 °C steps to identify primer dimers and to check the specificity of the reaction. Experiments were performed in triplicates and were normalised to the data of the small nuclear RNA (snRNA) U6, which was employed as an internal control. Relative miRNA expression levels were calculated using the comparative C_T ($\Delta\Delta C_T$) method as described in Section 3.3.2.

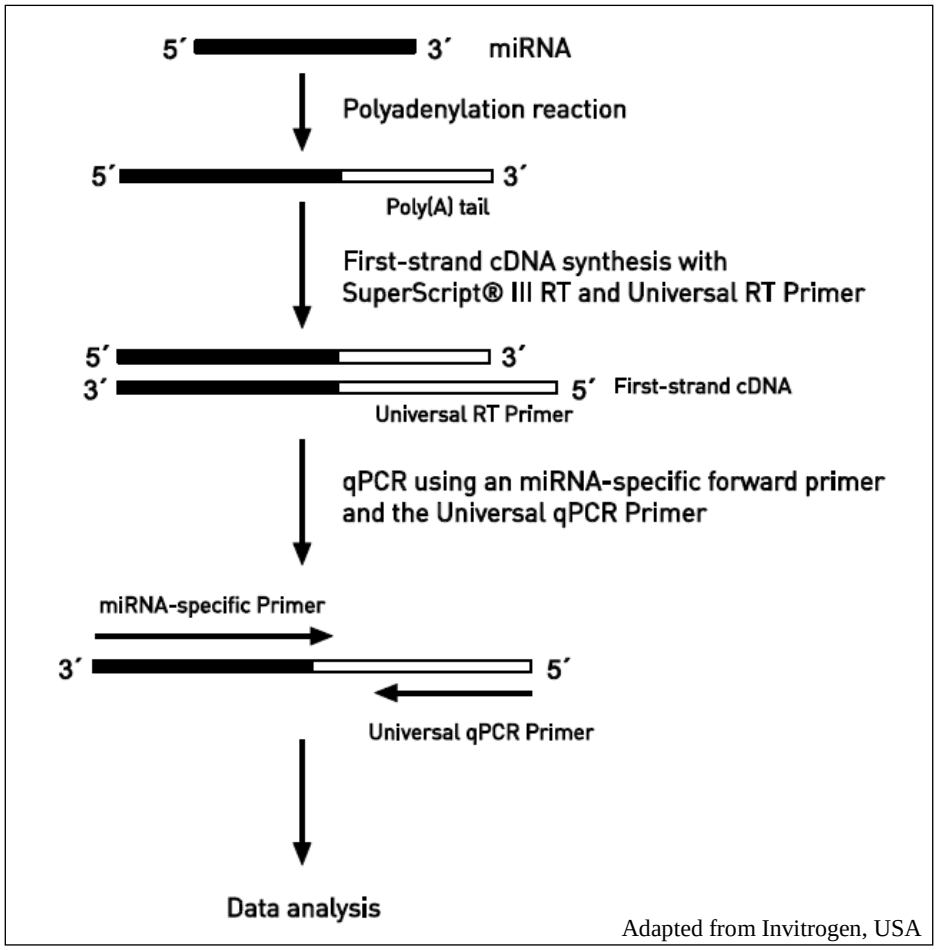


Figure 3.1: Workflow of miRNA real-time PCR.

Table 3.3: Setup of a miRNA real-time PCR reaction

PCR components	Volume per reaction	Final concentration/amount
2X SYBR GreenER qPCR SuperMix	10 µL	1X
Forward primer (10 µM)	0.4 µL	200 nM
Universal qPCR primer (10 µM)	0.4 µL	200 nM
cDNA template (5 ng/µL)	2 µL	10 ng
DEPC-treated water	7.2 µL	-
Total	20 µL	

3.6 Bioinformatics Analysis – miRNA Target Prediction and Gene Ontology Analysis

Online web-based miRNA target prediction algorithms were used to identify miRNA putative targets and their biological functions in the complex transcription factor – miRNA – mRNA regulatory network. The algorithms used included miRBase 19 (www.mirbase.org), TargetScanHuman 6.2 (www.targetscan.org), miRWalk (www.umm.uni-heidelberg.de/apps/zmf/mirwalk), miRanda (www.microrna.org), TarBase (diana.cslab.ece.ntua.gr), miRTarBase (mirtarbase.mbc.nctu.edu.tw) and RNAhybrid (bibiserv.techfak.uni-bielefeld.de/rnahybrid/). Criteria and parameters such as seed-sequence matching, conservation of miRNA binding sites in the targeted mRNA untranslated regions across various species and thermostability of miRNA:target duplexes were set to filter the large number of predicted targets to enhance the precision of miRNA target prediction. Gene ontology analysis was performed to further verify functions of the predicted targets by using the NCBI gene–gene ontology database (provided of Gene Ontology Annotation–UniProt-GOA) and the KEGG pathway database (www.genome.jp/kegg/pathway.html). Validated and putative target transcripts of miRNAs of interests were mapped onto selected pathways extracted from the KEGG pathway database to study if these miRNAs are associated with cancer-related pathways.

3.7 Real-Time PCR for Downstream Target messenger RNA (mRNA) Quantification

Total RNA was reversed transcribed to synthesise cDNA using the Phusion RT-PCR kit (Thermo Fisher Scientific, USA). One µg total RNA was first pre-denatured at 65 °C for 5 min with 0.5 mM dNTP mix and 100 ng oligo(dT) primer. Then, the reaction was added to a final volume of 20 µL with 1X RT buffer, RT enzyme mix (M-MuLV RNase H^+ Reverse Transcriptase and RNase inhibitor) and RNase-free water. The reaction tube was briefly spun down and incubated in a Veriti 96-well Thermal Cycler (Applied Biosystems, USA) at 25 °C for 10 min for primer extension, 40 °C for 30 min for cDNA synthesis and 85 °C for 5 min to terminate the reaction.

Similar to miRNA amplification, cDNA was subjected to mRNA quantification by real-time PCR using SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen, USA) (Table 3.4) under the same real-time PCR conditions as in miRNA real-time PCR, except that the primer annealing temperature in the 40 amplification cycles was modified to 60 °C for 1 min. Negative control was also included in the assay by substituting cDNA with DEPC-treated water to ensure no contaminant was detected in the PCR reactions. Experiments were performed in triplicates and were normalised to the C_T value of *GAPDH*. Relative mRNA levels were calculated using the comparative $\Delta\Delta C_T$ method.

Table 3.4: Setup of real-time PCR reaction for mRNA quantification

PCR components	Volume per reaction	Final concentration/amount
2X SYBR GreenER qPCR SuperMix	10 µL	1X
Forward primer (10 µM)	0.4 µL	200 nM
Reverse primer (10 µM)	0.4 µL	200 nM
cDNA template (5 ng/µL)	2 µL	10 ng
DEPC-treated water	7.2 µL	-
Total volume	20 µL	

3.8 Western Blot Analysis

3.8.1 Preparation of Buffers

Table 3.5: Preparation of buffers

Buffer	Methods of preparation
TGH buffer (1% Triton X-100, 10% Glycerol, 50 mM HEPES, pH 7.3)	A total of 5 mL Triton X-100 surfactant (Omnipur, EMD Chemicals, Merck KGaA, Germany), 50 mL Glycerol (Bio Basic Inc., Canada) and 25 mL 1 M HEPES (MP Biomedicals, USA) were dissolved and the volume was made up to 500 mL with distilled water. The solution was kept at 4 °C.
TGH with 1% Deoxycholate acid	An amount of 0.1 g Deoxycholate acid (Bio Basic Inc., Canada) was dissolved in 10 mL TGH buffer. The solution was kept at 4 °C.
50 mM N-Ethylmaleimide (NEM)	An amount of 62.5 mg NEM (Calbiochem, EMD Millipore, Merck KGaA, Germany) was dissolved in 10 mL TGH buffer. The solution was divided into small aliquots and kept at -20 °C.
0.5 M Ethylenediaminetetraacetic acid (EDTA)	An amount of 18.612 g EDTA (MP Biomedicals, USA) was added to 50 mL of distilled water, and the pH was adjusted to 8.0 with NaOH (MP Biomedicals, USA) to aid dissolving of EDTA in water. When dissolved, the volume was made up to 100 mL with distilled water and filtered with a 0.45 µm syringe filter (Sartorius AG, Goettingen, Germany). The solution was kept at 4 °C.
100 mM Ethylene glycol tetraacetic acid (EGTA)	An amount of 3.8 g EGTA (Calbiochem, Merck KGaA, Germany) was added to 20 mL distilled water, and the pH was adjusted to 8.0 with NaOH to aid dissolving of EGTA in water. When dissolved, the volume was made up to 100 mL with distilled water and filtered with a 0.45 µm syringe filter (Sartorius AG, Germany). The solution was kept at 4 °C.

Table 3.5 continued:

Buffer	Methods of preparation
1 M Sodium fluoride (NaF)	An amount of 4.198 g NaF (Merck KGaA, Germany) was dissolved in 100 mL distilled water. The solution was kept at 4 °C.
100 mM Sodium orthovanadate (Na_3VO_4)	This was prepared freshly as required. An amount of 18.4 mg Na_3VO_4 (Bio Basic Inc., Canada) was dissolved in 1 mL distilled water.
0.5 M Dithiothreitol (DTT)	An amount of 77.12 mg DTT (Bio Basic Inc., Canada) was dissolved in 1 mL distilled water. The solution was divided into each 200 µL aliquot and kept at -20 °C.
10 mg/mL Leupeptin	An amount of 5 mg Leupeptin (Bio Basic Inc., Canada) was dissolved in 500 µL distilled water. The solution was divided into small aliquots and kept at -20 °C.
10 mg/mL Aprotinin	An amount of 50 mg Aprotinin (Bio Basic Inc., Canada) was dissolved in 5 mL distilled water. The solution was divided into small aliquots and kept at -20 °C.
0.25 M Phenylmethylsulfonyl fluoride (PMSF)	An amount of 43.5 mg PMSF (Calbiochem, Merck KGaA, Germany) was dissolved in 1 mL Ethanol (Merck KGaA, Germany). The solution was divided into small aliquots and kept at -20 °C.
10% (w/v) Sodium dodecyl sulphate (SDS) solution	An amount of 10 g SDS (Omnipur, Merck KGaA, Germany) was dissolved in 90 mL distilled water. When dissolved, the volume was made up to 100 mL with distilled water and kept at room temperature.
10% (w/v) Ammonium persulfate (APS)	This was prepared freshly as required. An amount of 0.1 g APS (Merck KGaA, Germany) was dissolved in 1 mL distilled water.
1.5 M Tris, pH 8.8	An amount of 18.15 g Tris base (Norgen Biotek, Canada) was dissolved in 60 mL distilled water. When dissolved, pH was adjusted to 8.8 with 6 N HCl (Nacalai Tesque, Kyoto, Japan). The solution was then made up to 100 mL with distilled water and kept at 4 °C.

Table 3.5 continued:

Buffer	Methods of preparation
0.5 M Tris, pH 6.8	An amount of 6 g Tris base was dissolved in 60 mL distilled water. When dissolved, pH was adjusted to 6.8 with 6 N HCl. The solution was then made up to 100 mL with distilled water and the solution was kept at 4 °C.
SDS-PAGE running buffer (0.05 M Tris, 0.384 M Glycine, 0.1% SDS)	An amount of 12 g Tris base, 57.6 g Glycine (Merck KGaA, Germany) and 2 g SDS were dissolved in 500 mL distilled water. The volume was made up to 2 L with distilled water and kept at room temperature.
10X Tris-Buffered Saline (TBS) (0.5 M Tris, 1.5 M NaCl)	An amount of 60.57 g Tris base and 87.66 g NaCl (Bio Basic Inc., Canada) were dissolved in 500 mL distilled water. Then, pH of the solution was adjusted to 7.5 with 6 N HCl. The volume was made up to 1 L with distilled water and kept at room temperature.
Washing Buffer: 1X Tris-Buffered Saline with 0.05% Tween-20 (TBST)	To make 1X TBST, 100 mL concentrated 10X TBS was diluted to 1X by adding 900 mL distilled water. Then, 500 µL Tween-20 (Omnipur, Merck KGaA, Germany) was added to the diluted TBS and mixed well.
Blocking Buffer: Washing buffer with 3% milk	This was prepared freshly as required. An amount of 1.2 g non-fat milk powder (Bio Basic Inc., Canada) was dissolved in 40 mL 1X TBST.
1X Transfer Buffer/Towbin Buffer (25 mM Tris, 192 mM Glycine, 10% Methanol)	An amount of 3.03 g Tris base and 14.4 g Glycine were dissolved in 500 mL distilled water and the solution was then added with 200 mL Methanol (Merck KGaA, Germany). The volume was made up to 1 L with distilled water and kept at room temperature.
Mild Stripping Buffer	An amount of 1.5 g Glycine, 0.1 g SDS and 1 mL Tween-20 were dissolved in 50 mL distilled water. The pH of buffer was adjusted to 2.2 with 6 N HCl. The volume was then made up to 100 mL with distilled water and kept at room temperature.

Table 3.6: Preparation of TGH lysis buffer

Components	Volume (µL)	Final Concentration	Function
TGH with 1% Deoxycholate acid	752	-	Disrupting and dissociating protein interaction
50 mM NEM	200	10 mM	Deubiquitinase (DUB) inhibitor
0.5M EDTA	10	5 mM	Metallo-proteases that required Mg^{2+} and Mn^{2+}
100 mM EGTA	10	1 mM	Metalloproteases that required Ca^{2+}
1 M NaF	10	10 mM	Serine/Threonine phosphatase inhibitor
100 mM Sodium orthovanadate	10	1 mM	Tyrosine phosphatase inhibitor
0.5 M DTT	2	1 mM	Redox reagent
10 mg/mL Aprotinin	1	10 µg/mL	Protease inhibitor (Trypsin, Chymotrypsin, Plasmin)
10 mg/mL Leupeptin	1	10 µg/mL	Protease inhibitor (Lysosomal)
0.25 M PMSF	4	1 mM	Protease inhibitor (Cysteine, Serine)
Total	1000 µL		

3.8.2 Preparation of Cell Lysates and Quantification of Protein Content in the Lysates

Crude protein lysates were prepared from the four colon cancer cell lines by lysing the cells in TGH lysis buffer (Table 3.6). The solubilised proteins isolated from colon cancer cells were then migrated individually through a separating gel. Two commercially purchased crude protein lysates which are non-cancerous colonic tissues derived from two donors, were included in this study as normal.

A T-25 cell culture flask with 90% cell confluency (approximately 5 x 10^6 cells) was placed on ice and the spent medium was removed by aspiration. The cells were washed twice with ice-cold PBS and the PBS was discarded. A volume of 500 µL TGH lysis buffer was added to the adherent cells and the cells were scraped off the flask using an ice-cold plastic cell scrapper (TPP, Switzerland). The cell suspension was gently transferred into a pre-cold microfuge tube and was incubated in the lysis buffer for 15 min on ice with agitation on a gyratory rocker (Stuart Scientific, UK). The cells suspension was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was aspirated and removed to a fresh microfuge tube on ice. The cell pellet was discarded.

Protein concentration was determined by Quick Start Bradford Protein Assay (Bio-Rad, USA) in order to standardise the amount of protein loaded into the gel. The assay was carried out in a 250 µL microplate assay format. A protein standard curve was constructed based on serial dilution of concentrated bovine serum albumin (BSA) in a linear range from 125 to 2,000 µg/mL. Five µL of each of the diluted protein standards and 5X diluted unknown samples were pipetted into the wells in triplicates. Then, 250 µL 1X Dye Reagent (Bio-Rad, USA) was added to each well, mixed and incubated for 5 min. The amount of protein in the microplate was then measured at absorbance of 595 nm in a microplate reader (Infinite M200, Tecan, USA). The concentration of the sample protein lysate was determined based on the average absorbance reading of the protein lysate and the BSA protein standard curve plotted.

3.8.3 Protein Separation by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A Mini-PROTEAN Tetra Cell system (Bio-Rad, USA) was used in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A short plate was placed on top of a spacer plate (1.0 mm thickness) and they were slide into a casting frame to form a gel cassette assembly. The gel cassette assembly was secured on a casting stand.

A 10% resolving gel was prepared from 2.5 mL of bis/acrylamide [40% (w/v) T, 3% C, PlusOne ReadySol IEF, GE Healthcare, UK], 2.5 mL 1.5 M Tris (pH 8.8), 100 µL 10% SDS and 4.85 mL distilled water and mixed well. Five µL of tetramethylethylenediamine (TEMED, Omnipur, Merck KGaA, Germany) and 50 µL 10% APS were lastly added to the resolving gel solution. All the components were mixed well and poured in between the gel cassette glass plates assembled on the casting stand. The resolving gel was gently overlaid with 100 µL of 1-butanol (Merck KGaA, Germany) to shield the gel from air. Butanol was rinsed off with distilled water when the resolving gel had polymerised. Water residue and butanol were removed with the edge of tissue paper. A 5% stacking gel was prepared from 0.5 mL 40% (w/v) bis/acrylamide, 1 mL 0.5 M Tris, pH 6.8, 40 µL 10% SDS and 2.07 mL distilled water and mixed well. A total of 3.3 µL TEMED and 30 µL 10% APS were then added to the stacking gel solution and mixed well. The stacking gel solution was layered directly onto the polymerised resolving gel. A 10-well 1.0-mm comb was carefully inserted into the stacking gel layer without forming air bubbles. The comb was removed after the stacking gel polymerised and the formed wells were rinsed with running buffer. The two-gel cassette sandwiches with the short glass plates facing inward were clamped onto the electrode assembly and placed into Mini-PROTEAN Tetra Tank. If only one gel cassette sandwich was prepared, the buffer dam was used to create a functioning assembly. The upper chamber (assembly) was filled with SDS-PAGE running buffer until full and lower buffer chamber was filled until the indicated level of 550 mL for 2 gels. Then, 35 µg protein lysate was mixed with an equal volume of 2X Laemmli Sample Buffer (Sigma-

Aldrich, USA) and the mixture was briefly spun and denatured at 95 °C for 5 min in a block heater (Stuart Scientific, UK). Aliquots of denatured protein sample buffer mixture and 4 µL Full-Range Rainbow Molecular Weight Markers (GE Healthcare, UK) were loaded into the wells accordingly. The gel tank was attached to a power supply at a constant voltage of 120 V and running time was set for 75 min.

3.8.4 Semi-Dry Transfer of Protein from Gel to Nitrocellulose Membrane

When the dye front reached almost the bottom of the resolving gel, the power supply was turned off. The electrophoresis apparatus was disassembled and the gel cassette was removed. The gel was removed gently from the gel cassette by separating the two glass plates. The stacking-gel layer was removed. The resolving gel was then transferred to a container containing 1X Transfer Buffer (Table 3.5) and was equilibrated for 15 min. Before termination of electrophoresis, Hybond ECL Nitrocellulose Membrane (GE Healthcare, UK) and Hybond Blotting Paper (GE Healthcare, UK) were cut to the dimension of the gel, approximately 10 cm x 6.5 cm. Nitrocellulose membrane and four pieces of blotting papers were soaked and pre-equilibrated in 1X Transfer Buffer together with the resolving gel.

The SDS-PAGE gel with the resolved protein samples was subjected to semi-dry electro-transfer by using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell without prior staining (Bio-Rad, USA). A transfer sandwich was arranged accordingly. Two pieces of equilibrated blotting papers were placed on the platinum anode of the semi-dry transfer cell, followed by equilibrated nitrocellulose membrane, then equilibrated polyacrylamide gel and finally another two pieces of blotting papers. Trapped bubbles were removed using a disposable serological pipette as a roller. The upper cathode was placed on top of the transfer sandwich. The separated protein samples were transferred and blotted onto nitrocellulose membrane at a constant voltage of 15 V for 15 min. Then, the transferred membrane was removed from the transfer sandwich and the gel orientation was marked on the membrane with a pencil. The membrane was washed twice with distilled water for 5 min each with gentle agitation on a gyratory rocker. The membrane was stained with RedAlert Stain (Novagen, Merck KGaA, Germany) to verify the transfer of proteins before western blot analysis. The stain was reversed by washing the membrane in distilled water. The membrane was then ready for blocking.

3.8.5 Membrane Blocking

The transferred membrane was blocked with milk to prevent non-specific background and binding of the primary or secondary antibodies onto the membrane. The membrane was blocked or incubated in 10 mL of blocking buffer (Table 3.5) for 2 h at room temperature with gentle agitation on a

gyratory rocker. The blocking solution was discarded and the membrane was washed twice with 10 mL washing buffer (Table 3.5) for 5 min with gentle agitation on a gyratory rocker, with fresh changes of washing buffer.

3.8.6 Antibody Staining

After the washing step, the membrane was probed with 10 mL primary mouse monoclonal antibody against KRAS (Upstate, EMD Millipore, Merck KGaA, Germany) diluted at 1:2000 with blocking buffer and incubated overnight with slight agitation at 4 °C. Expression of IGF1R protein was detected with 10 mL primary mouse monoclonal antibody against IGF1R (Upstate, EMD Millipore, Merck KGaA, Germany) diluted at 1:2000 with washing buffer and incubated overnight with slight agitation at 4 °C. The membrane was washed with 10 mL of washing buffer with agitation on a gyratory rocker for 10 min and the step was repeated twice.

The membrane was then incubated in 10 mL horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal secondary antibody (Genetex Inc., USA) diluted at 1:10000 with blocking buffer and incubated for 1.5 h with gentle agitation at room temperature. The membrane was again washed with 10 mL washing buffer with agitation on a gyratory rocker for 10 min and the step was repeated twice.

GAPDH (Abcam, UK) was used as a loading control to ensure that different protein samples were equally loaded to each lane in the same SDS-PAGE gel and were evenly transferred to the nitrocellulose membrane. Equal amounts of protein samples loaded are crucial especially when a comparison was to be made between the protein expression levels in different samples.

3.8.7 Chemiluminescence Detection

The excessive wash buffer was drained off from the washed membrane blot and the protein side of the membrane was placed facing upward on a sheet of SaranWrap. Equal volume of Detection Solutions 1 and 2 supplied in the Amersham Enhanced chemiluminescent (ECL) Western Blotting Detection Reagent (GE Healthcare, UK) were mixed and were added directly to the protein side of the membrane. The membrane was incubated for 2 min at room temperature. Cumulative chemiluminescent signal emanating from the membrane after an exposure time of 15 min was captured by a CCD camera of the BioSpectrum Imaging System (Ultra-Violet Products, Cambridge, UK) and the image was saved in a computer for further analysis.

3.8.8 Stripping and Reprobing

After chemiluminescence detection, the membrane was rinsed with washing buffer and the primary and secondary antibodies were stripped from

the developed membrane. The washed membrane was then incubated in 10 mL mild stripping buffer (Table 3.5) twice for 10 min at room temperature. The membrane was then washed twice with 10 mL PBS for 10 min. The membrane was washed again with 10 mL washing buffer twice for 5 min. Subsequently, the stripped membrane was blocked in 10 mL blocking buffer for 2 h at room temperature with agitation and was then rinsed with washing buffer. The membrane was incubated in 10 mL mouse monoclonal antibody diluted at recommended ratio stated in the protocol with blocking buffer and incubated for 2 h with agitation at room temperature. This was followed by washing step, secondary-antibody staining and further washing step as described in Section 3.8.6. Chemiluminescent signal detection was performed as described in Section 3.8.7.

3.8.9 Western Blot Densitometric Analysis

Densitometric analysis of the protein bands was performed using the Area Density Analysis Tool in the VisionWorks®LS Image Acquisition and Analysis Software (Ultra-Violet Products, UK) to measure the relative intensities of protein bands developed in the western-blot membrane. The band intensities were used to quantify the expression levels of the proteins of interest in colon cancer cells relative to cells of normal colon tissues after the band intensity values were normalised to those of the GAPDH loading control.

3.9 Transient Transfection with Synthetic miRNAs

HCT-15 colon cancer cells were seeded to a 6-well plate (TPP, Switzerland) at a density of 5 x 10^5 cells per well on Day 0. On Day 1, 90 pmol mirVana miRNA inhibitor, or miRNA mimic, or miRNA inhibitor or mimic negative control (Applied Biosystems, USA) was diluted in 250 µL serum-free DMEM medium, then, 9 µL Lipofectamine RNAiMAX Reagent (Invitrogen, USA) was also diluted in 250 µL serum-free DMEM medium. Both diluted synthetic miRNAs and Lipofectamine RNAiMAX reagent were mixed and the mixture was incubated for 20 min at room temperature. The synthetic miRNA-lipofectamine reagent complex was then added to the cells seeded on Day 0 and the cells were incubated for another 48 h in a 37 °C CO_2 incubator. Non-treated HCT-15 cells supplemented with 500 µL serum-free DMEM (with 9 µL Lipofectamine RNAiMAX Reagent) were included as a mock control in the assay. After 48 h of transfection (Day 3), the cells were harvested for preparation of RNA or protein lysates for downstream analyses to determine changes of miRNA, mRNA and protein expression levels before and after the cells were transiently transfected with the synthetic miRNAs.

3.10 Statistical Analysis

Statistical analysis was performed in SPSS for Windows v11.5. Real-time PCR results were reported as average of $\log_2$(fold change) ± standard deviation (SD). Western blot analysis results were reported as average of fold

difference ± SD. Data were analysed by Student's *t*-test (two-tailed distribution) comparing the differences of miRNA, mRNA and protein expression levels between colon cancer cells and non-cancerous colonic tissues. Statistical significance was accepted at $p < 0.05$.

3.11 Flow Chart of Methodology

Flow chart of methodology for this study was showed in Figure 3.2.

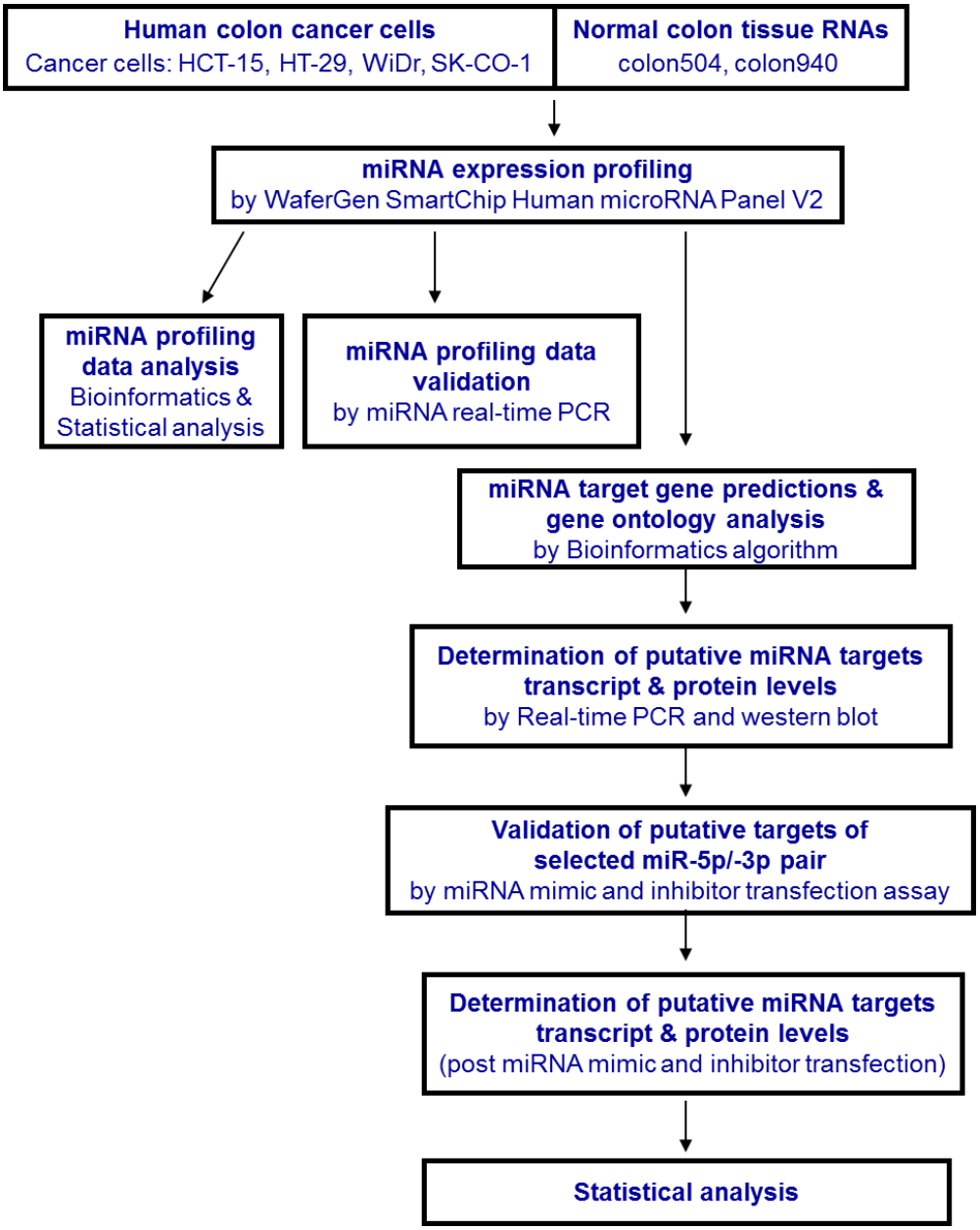


Figure 3.2: Flow chart of methodology.

CHAPTER 4

RESULTS

4.1 Colon Cancer Cell Culture and RNA Preparation

Among 23 types of colon originated colorectal adenocarcinoma cell lines available from ATCC, four cell lines (HCT-15, HT-29, SK-CO-1 and WiDr) were used to investigate dysregulation of miRNAs in colon cancer cells.

RNAs were isolated from the four colon cancer cell lines. Concentration, purity and integrity of the isolated RNA samples were determined to ensure that the RNAs were qualified for global miRNA profiling analysis (Table 4.1 and Figure 4.1). The A_{260}/A_{280} ratios of all the isolated RNA samples were 1.8 or higher indicating high purity; A_{260}/A_{230} ratios were 2.0 or higher indicating the samples were free from impurities carried over from RNA isolation (Table 4.1). The RNA yields (Table 4.1) were also sufficient for miRNA profiling analysis and further analyses.

Agarose gel electrophoresis analysis in Figure 4.1 showed that the 28S and 18S ribosomal RNA (rRNA) bands appeared sharp and intense, and the intensity of the 28S band was approximately twice that of the 18S band,

indicating high quality and intact RNA samples. Besides, the small molecular weight RNA species (miRNAs, tRNAs, snRNAs, 5S and 5.8S rRNAs) with less than 200 nucleotides (nt) were separated from the higher molecular weight RNA species (18S, 28S rRNAs and mRNAs) (Figure 4.1). The bands of small molecular weight RNA were intact and intense which indicated that the small RNA fractions were successfully retained in the isolated RNA samples.

Table 4.1: Purity and concentration of RNA samples isolated from colon cancer cell lines

Cell lines	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µL)	Yield (µg)
HCT-15	1.955	2.189	1318	46.13
HT-29	2.022	2.197	1630	57.05
SK-CO-1	1.878	2.000	1764	61.74
WiDr	1.928	2.095	1504	52.64

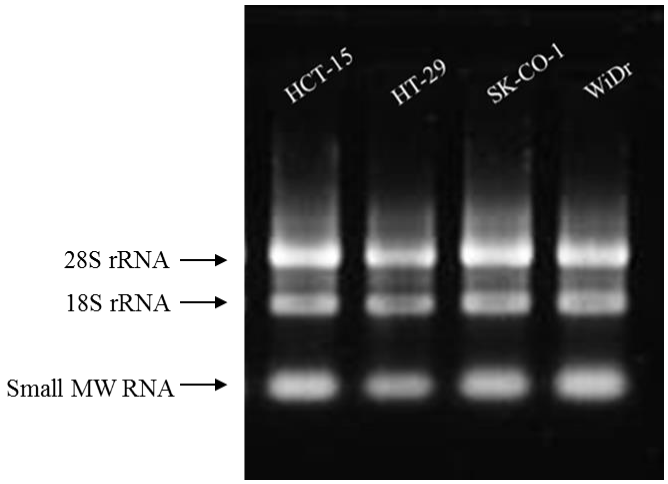


Figure 4.1: RNA integrity assessment by agarose gel electrophoresis. One µL of total RNAs isolated from HCT-15, HT-29, SK-CO-1 and WiDr cells was subjected to 1% agarose gel electrophoresis at 80 V for 45 min. The gel was stained with 3X GelRed nucleic acid stain and visualised by exposure to 302 nm UV light on a UVP BioSpectrum Imaging System. MW: molecular weight.

4.2 Global miRNA Expression Profiling in Human Colon Cancer Cells

To systematically investigate miRNA dysregulation in colon cancer cells, total RNA samples of colon cancer cell lines HCT-15, HT-29, SK-CO-1 and WiDr, together with two normal colon tissue RNA samples were subjected to global miRNA expression profiling analysis using WaferGen SmartChip Human MicroRNA Panel V2, a nanoscale miRNA real-time PCR array (see Materials and Methods section 3.3.1). Data analyses identified a total of 128 (10.8%) differentially expressed miRNAs out of the 1,190 miRNAs included in the profiling panel, in at least two or more of the four colon cancer cell lines, relative to the two normal colon tissues used.

Volcano plot analysis (Figure 4.2) enables quick visual identification of differentially expressed miRNAs in colon cancer cells by sorting out the miRNAs with statistically significant differential expression ($p < 0.05$) and significant $\log_2$(fold change) values with a cut-off point set at 1.5. The differentially expressed miRNAs were presented as green or red dots above the blue line in the Volcano plot. Amongst the differentially expressed miRNAs, 92 miRNAs (71.9%) were significantly up-regulated (red dots above the blue line) and they were recognised as putative oncogenic miRNAs. Thirty six miRNAs (28.1%) were down-regulated (green dots above the blue line) and were probably tumour suppressive miRNAs. Our data, therefore, suggest significant miRNA dysregulation in colon carcinogenesis, an observation

supported by previous reports (Sarver et al., 2009; Slaby et al., 2009; Panarelli and Yantiss, 2011; Wu et al., 2011).

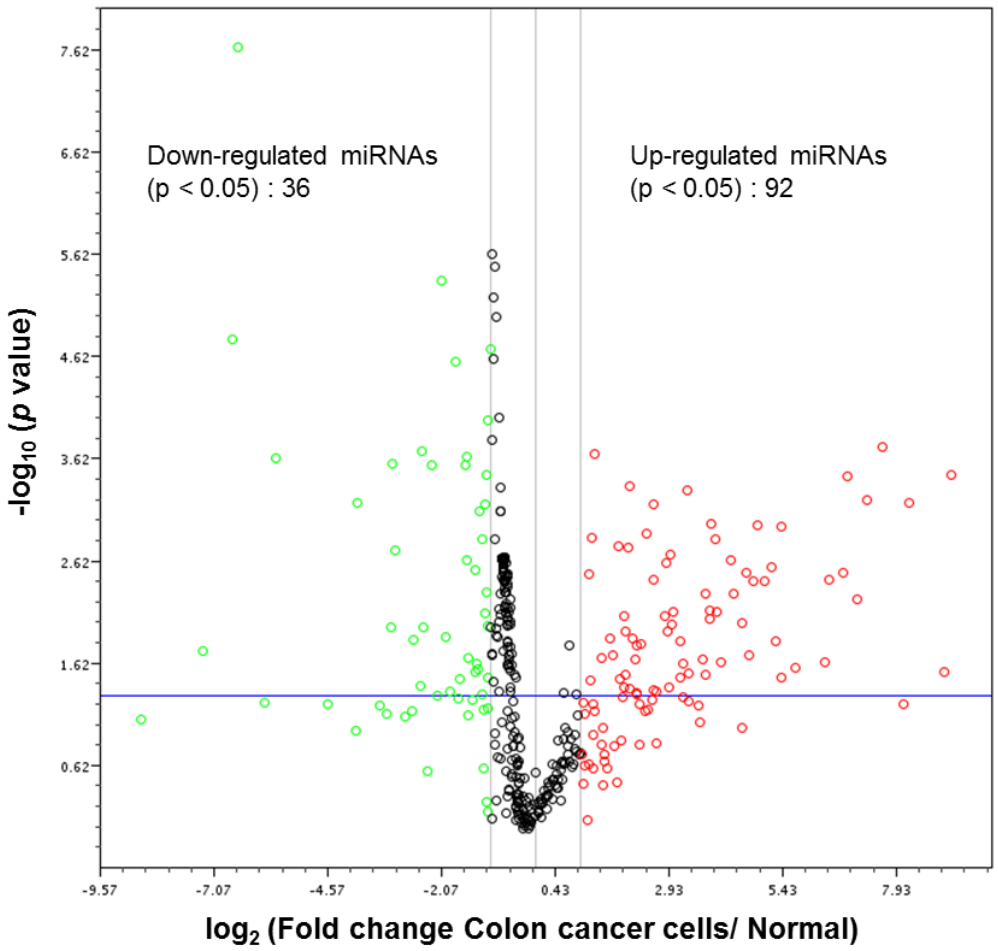


Figure 4.2: Volcano plot analysis of miRNA profiling of colon cancer cells. The analysis was performed using the miScript miRNA PCR Array Data Analysis Web Portal. miRNAs with statistically significant differential expression ($p < 0.05$) lie above the blue horizontal threshold line. miRNA with significant $\log_2$(fold change) values ($-1.5 < \log_2FC > 1.5$) lie outside a pair of grey vertical threshold lines. Green dots indicate minimum level of miRNA expression ($\log_2FC < -1.5$); black dots indicate average or weak miRNA expression; red dots indicate strong or maximum level of miRNA expression ($\log_2FC > 1.5$). FC: Fold change.

Hierarchical clustering analysis of the miRNA profiling was performed to depict expression of the dysregulated miRNAs in the four colon cancer cells (Figure 4.3). The clustering analysis demonstrated separation of normal from cancer cells although the separation was incomplete. miRNAs were organised according to the similarity or dissimilarity of expression profiles, and hence, miRNAs with similar expression profiles were grouped together in the clustergram. Dendrograms in the clustergram showed the relatedness of the expression profiles between individual miRNAs or the relatedness of the miRNA expression profiles between individual samples. In the clustergram, samples were grouped into two clusters: cluster 1 represents normal tissue samples colon 504 and 940 and cluster 2 represents colon cancer cells. The long horizontal dendrogram arm between the normal and cancer clusters indicates major differences in the miRNA expression profiles between normal tissues and cancer cells. The four colon cancer cell lines were further grouped into two sub-clusters: sub-cluster 1 includes HT-29 and WiDr cells, and sub-cluster 2 includes HCT-15 and SK-CO-1 cells indicating similar miRNA expression of colon cancer cells within each sub-clusters.

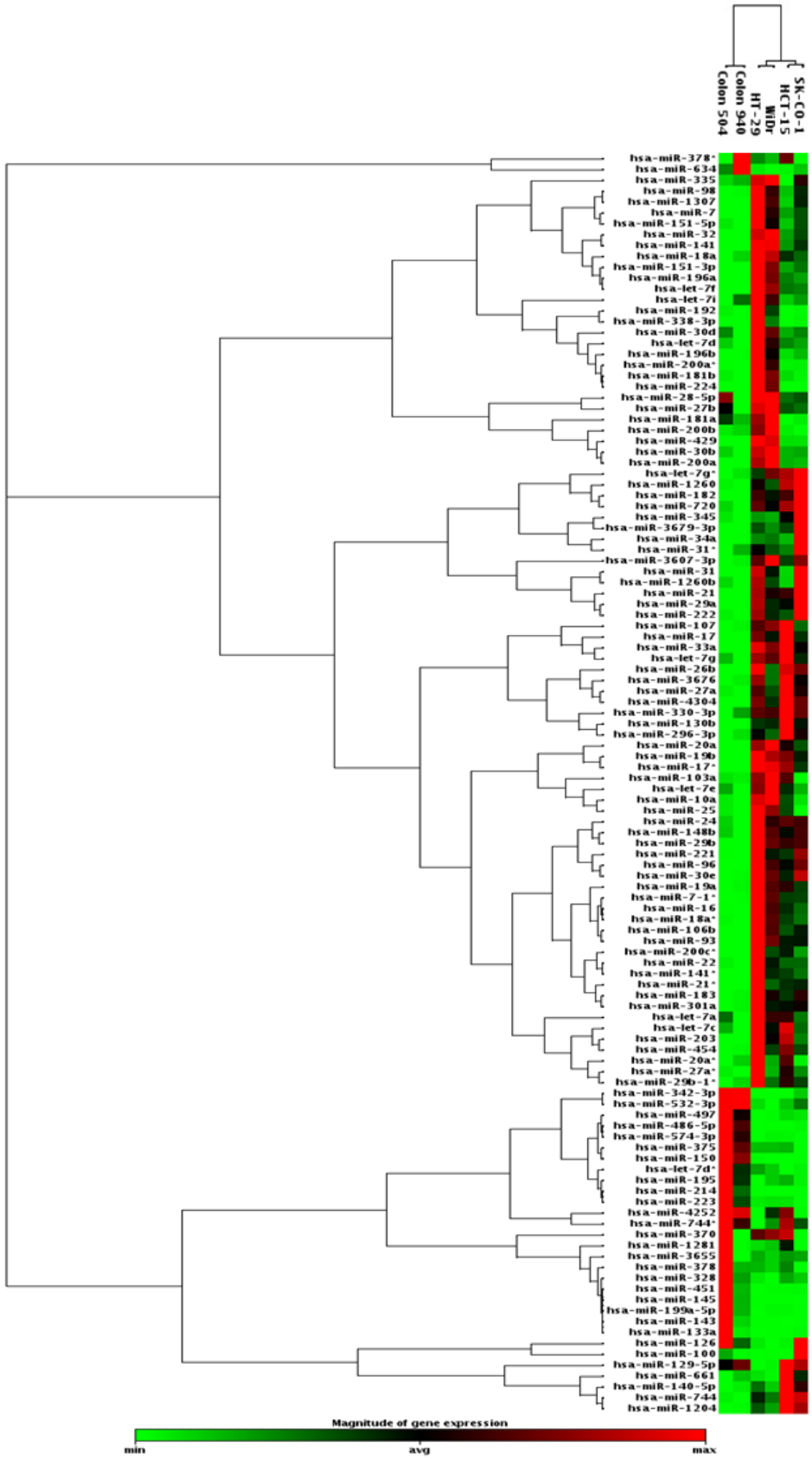


Figure 4.3: Hierarchical clustering analysis of miRNA profiling of colon cancer cells. The analysis was performed using the miScript miRNA PCR Array Data Analysis Web Portal. A colour bar code indicating the magnitude of miRNA gene expression is shown at the bottom**.**

4.3 miRNA Expression Profiling Analysis Reveals miRNAs that are Activated and Shutdown in Colon Cancer Cells

Amongst the up- and down-regulated miRNAs, 19 miRNAs were found to be activated in colon cancer cells but not expressed in cells of normal tissues, 12 were shutdown in colon cancer cells as they were originally expressed in normal cells (Tables 4.2 and 4.3). Activated miRNAs are defined as miRNAs that are only detected in colon cancer cells at $C_T \leq 30$ (a cut-off C_T value set by WaferGen Biosystems, the platform designer), but not in normal cells. Shutdown miRNAs refer to miRNAs only detected in normal cells at $C_T \leq 30$ but not in cancer cells ($C_T > 30$). The fold changes observed in the cancer cells relative to the cells of normal tissues as shown in Table 4.3 ranged from significantly activated (up-regulated) by 7.19 ± 0.78 fold in miR-20a-5p to shutdown (down-regulated) by -8.66 ± 0.16 fold in miR-3195. Amongst the 31 activated and shutdown miRNAs, 11 of them (32%) have not yet been reported in the literature related to colon cancer and most of them are not yet validated or are miRNAs annotated only recently (Table 4.3). Twenty one of the activated and shutdown miRNAs (68%) are miRNAs that have previously been reported to be associated with colon cancer suggesting the possibility of making these activated miRNAs as potential targets for the development of diagnostic markers for colon cancer as they are detected in high steady level in colon cancer, but very low abundance or not detectable in normal cells.

Table 4.2: Differentially expressed miRNAs in colon cancer cells

	Up-regulated miRNA		Down-regulated miRNA	
	Cancer & normal cells	**Cancer cells only (Activated)[1]**	**Cancer & normal cells**	**Normal cells only (Shutdown)[2]**
Number of miRNA	73	19	24	12
	92 (71.9%)		36 (28.1%)	
Total	128			

Data include only miRNA species detected in two or more cancer cell lines and the average $\log_2$(fold change) cut-off point was set at 1.5, $p < 0.05$. [1]Activated miRNAs refer to miRNAs only detected in cancer cells at $C_T \leq 30$ but not in normal cells. [2]Shutdown miRNAs refer to miRNAs only detected in normal cells at $C_T \leq 30$ but not in cancer cells.

Table 4.3: miRNAs that are activated or shutdown in colon cancer cells relative to normal colon cells

miRNA family	miRNA[1]	log_2(fold change)[2] (Mean ± SD)	Predicted/Validated targets[4]	Function/Phenotype(s) affected[4]
			(a) Activated in colon cancer cells (n = 19)	
let-7	miR-98-5p	4.45 ± 1.33**	*HMGA2*[#], *TP53*, *FAS*, *IGF1*	Apoptosis, cell cycle, EMT, p53, MAPK, PI3K-Akt signalling pathway
mir-7	miR-7-5p[5]	6.44 ± 1.14**	*IGF1R*[#], *CCND1*, *EGFR*[#]	Apoptosis, cell cycle, p53, MAPK, PI3K-Akt signalling pathway
mir-8	miR-200a-5p[5]	3.74 ± 2.77	*TIAM2*, *ZEB2*, *FGF4*	Apoptosis, EMT, MAPK, PI3K-Akt signalling pathway
mir-8	miR-200c-5p[5]	3.93 ± 1.05**	*SPRY2*, *CREBBP*	Apoptosis, cell cycle
mir-8	miR-429	5.27 ± 2.39*	*IGF1*, *SOX2*, *ZEB1/2*[#], *KRAS*	Apoptosis, cell cycle, EMT, p53, MAPK, PI3K-Akt signalling pathway
mir-17	miR-20a-5p	7.19 ± 0.78**	*E2F1*[#], *PTEN*[#], *TCF7L1*, *KRAS*, *CDKN1A*[#]	Apoptosis, cell cycle, EMT, p53, PI3K-Akt signalling pathway
mir-17	miR-20a-3p	1.58 ± 0.98	*KRAS*, *PCNA*	Apoptosis, cell cycle, MAPK, PI3K-Akt signalling pathway
mir-19	miR-19a-3p	3.61 ± 0.64**	*PTEN*[#], *SMAD4*[#], *BCL2L11*[#]	Apoptosis, cell cycle, EMT, p53, PI3K-Akt signalling pathway
mir-21	miR-21-3p	5.99 ± 0.85**	*CASP7*, *TGFB2*, *IKBKB*	Apoptosis, cell cycle, EMT, MAPK signalling pathway
mir-29	miR-29b-1-5p[5]	2.93 ± 0.84**	*BIRC2*, *TCF7L2*	Apoptosis, cell cycle, EMT
mir-32	miR-32-5p	4.80 ± 1.17**	*BCL2L11*[#], *CDKN1C*, *KLF4*, *PTEN*, *FASLG*, *MAP2K4*	Apoptosis, cell cycle, EMT, p53, MAPK, PI3K-Akt signalling pathway

Table 4.3 continued:

miRNA family	miRNA[1]	log_2(fold change)[2] (Mean ± SD)	Predicted/Validated targets[4]	Function/Phenotype(s) affected[4]
		(a) Activated in colon cancer cells (n = 19)		
mir-130	miR-301a-3p[5]	3.32 ± 0.58**	*SERPINE1[#], AXIN2, ATM, TGFBR2, IGF1*	Apoptosis, cell cycle, EMT, p53, MAPK, PI3K-Akt signalling pathway
mir-224	miR-224-5p	6.74 ± 3.90	*SMAD4[#], ADAM18*	Cell cycle, EMT
mir-335	miR-335-5p	2.67 ± 1.39	*BCL2L2[#], RB1[#], KRAS*	Apoptosis, cell cycle, MAPK, PI3K-Akt signalling pathway
mir-454	miR-454-3p	3.56 ± 0.78**	*SMAD4[#], ATM, AXIN2, TGFBR2, IGF1*	Apoptosis, cell cycle, EMT, p53, MAPK, PI3K-Akt signalling pathway
mir-1204	miR-1204	2.66 ± 0.93**	*PDE3A, MAPK13*	Apoptosis, cell cycle, MAPK signalling pathway
-	miR-1274a[3, 5]	5.77 ± 1.01**	*MAP3K1, CDKN2B, VEGFA*	Apoptosis, cell cycle, MAPK, PI3K-Akt signalling pathway
-	miR-3679-3p[5]	3.39 ± 0.79**	*TNFSF10, SMAD2*	Apoptosis, cell cycle
-	miR-4304[5]	6.14 ± 0.55**	*PSMD1, SERPINB13*	Apoptosis, cell cycle
		(b) Shutdown in colon cancer cells (n = 12)		
mir-15	miR-195-5p	-2.11 ± 0.0008**	*BCL2[#], AXIN2, CCND1[#], RAF1[#], SIAH1, IGF1*	Apoptosis, cell cycle, EMT, p53, MAPK, PI3K-Akt signalling pathway
mir-143	miR-143-3p	-8.00 ± 2.74**	*KRAS[#], MAPK7[#]*	Apoptosis, cell cycle, MAPK, PI3K-Akt signalling pathway
mir-145	miR-145-5p	-5.24 ± 2.16**	*TGFBR2, SMAD3, CDKN1A[#]*	Apoptosis, cell cycle, EMT, p53, MAPK, PI3K-Akt signalling pathway

Table 4.3 continued:

miRNA family	miRNA[1]	log_2(fold change)[2] (Mean ± SD)	Predicted/Validated targets[4]	Function/Phenotype(s) affected[4]
		(b) Shutdown in colon cancer cells (n = 12)		
mir-199	miR-199a-5p	-3.84 ± 2.16**	*IKBKB*[#], *CAV1*[#], *GSK3B*	Apoptosis, MAPK signalling pathway, EMT
mir-223	miR-223-3p	-2.83 ± 0.001**	*IGF1R*[#], *FGFR2*, *SEPT6*, *FOXO1*[#], *FOXO3*	Apoptosis, cell cycle, MAPK signalling pathway
mir-342	miR-342-3p	-6.87 ± 0.24**	*BMP7*[#], *MYLK2*, *VEGFA*	Apoptosis, cell cycle, EMT, MAPK, PI3K-Akt signalling pathway
mir-370	miR-370-3p	-6.83 ± 0.003**	*BAX*, *MAP3K8*[#]	Apoptosis, cell cycle, p53, MAPK signalling pathway
mir-378	miR-378a-5p	-2.60 ± 0.003**	*DCC*, *GABPA*	Apoptosis
mir-451	miR-451a	-2.70 ± 2.06*	*MIF*, *MYC*[#]	Apoptosis, cell cycle, MAPK, PI3K-Akt signalling pathway
mir-1268	miR-1268a[5]	-3.51 ± 0.26**	*MYLK2*, *MAP4K1*	Cell cycle, MAPK signalling pathway
-	miR-3195[5]	-8.66 ± 0.16**	*TIE1*, *ERCC3*	Angiogenesis, apoptosis, cell cycle
-	miR-4252[5]	-4.84 ± 0.33**	*PDCD10*, *FAS*	Apoptosis, p53, MAPK signalling pathway

Data in each group are presented in increasing order of miRNA family name. [1]Nomenclature used is according to miRBase 19. [2]Data shown are limited to altered expression of miRNA in two or more colon cancer cell lines relative to normal colon tissues with log_2(fold change) > 1.5 or < -1.5. *p < 0.05 and **p < 0.01. [3]The putative mature miR-1274a sequence appears to be a fragment of a lysine tRNA (Schopman et al., 2010). [4]Data of putative targets and functions are derived from various miRNA target gene prediction algorithms and gene ontology databases described in Materials and Methods Section 3.6. Hash (#) indicates validated target. [5]miRNAs that not reported in colon cancer related literature.

4.4 Target Transcripts, Putative Biological Functions of Activated and Shutdown miRNAs

Previous researches have already revealed general involvement of miRNA in colon cancer without categorising differentially expressed miRNAs into activated and shutdown miRNA species (Sarver et al., 2009; Gregersen et al., 2010; Parasramka et al., 2012; Zhang et al., 2012). Therefore, to study biological functions of target transcripts of the thirty one activated and shutdown miRNAs and to further elucidate the potential cancer-related regulatory pathways that may be regulated by these miRNAs, miRNA target gene prediction algorithms and gene ontology database were used as described in Materials and Methods Section 3.6. The data (Table 4.3) showed that most of target transcripts of these 31 miRNAs, either predicted or validated target transcripts are involved in specific cancer-related regulatory pathways: 29 miRNAs (93.5%) are apoptosis-related, 27 miRNAs (87.1%) are cell cycle-related, 22 miRNAs (70.9%) are MAPK signalling pathway-related, 17 miRNAs (54.8%) are PI3K-Akt signalling pathway-related, 15 miRNAs (48.4%) are epithelial-mesenchymal transition (EMT)-related and 12 miRNAs (38.7%) are p53 signalling pathway-related (Table 4.4). Besides, it is further observed that most of the activated miRNAs (94.7%, 18 miRNAs) are involved in apoptosis and cell cycle regulatory pathways; most of the shutdown miRNAs (91.7%, 11 miRNAs) are strongly involved in MAPK signalling pathway (Table 4.3). The data indicate that the activated and shutdown miRNAs identified in this study in colon cancer cells are engaged in

multiple cancer-related pathways and played crucial roles in regulation of various key cellular processes involved in carcinogenesis.

Table 4.4: Putative target transcripts of activated and shutdown miRNAs[1] involved in cancer-related regulatory pathways

Process	Total number of miRNAs
Apoptosis	29 (93.5%)
Cell cycle	27 (87.1%)
MAPK signalling pathway	22 (70.9%)
PI3K-Akt signalling pathway	17 (54.8%)
EMT	15 (48.4%)
p53 signalling pathway	12 (38.7%)

Data shown above are the summary of Table 4.3; [1]n = 31.

The data revealed that target transcripts of 18 miRNAs (58%) of the activated and shutdown miRNAs were validated targets (Table 4.3). The validated miRNAs, either miRNA-5p or 3p, are expected to be the guiding strand miRNA species that are accumulated in significant higher steady-state level in cells. Thirteen miRNAs (42%) of activated and shutdown miRNAs with predicted and not yet experimentally validated targets are most likely the passenger strand miRNA species (or previously known as miRNA* species) that usually expressed in lower abundance or are miRNAs annotated only recently with nomenclature numbering more than four digit number.

Members from the same miRNA family are revealed to possess the similar function and regulate the same pathway by targeting on the same transcript. As shown in Table 4.3, *ZEB2* is predicted to be targeted by both miR-200-5p and miR-429 from the mir-8 family. *KRAS* transcript is predicted to be targeted by miR-20a-5p and miR-20a-3p, which is encoding for protein involved in the apoptosis, MAPK and phosphatidylinositol-3-kinase-Akt (PI3K-Akt) signalling pathway. These two miRNAs are derived from the same miRNA precursor which are also originated from the same family and may possess similar biological function in cells.

Besides, multiple miRNAs are revealed to target the same target transcript and regulate the same pathway. As shown in Table *BCL2L11* transcript is targeted by miR-19a-3p and miR-29b-1-5p and they are from

different miRNA families. *SMAD4* transcript is also targeted by multiple miRNAs from different families, miR-454-3p, miR-224-5p and miR-19a-3p, involved in the EMT and cell cycle pathways. Moreover, the same miRNA gene is observed cross targeting on different transcripts encoding for proteins involved in the same regulatory pathway. For example, *EGFR* and *IGF1R* are transcripts encoding for proteins involved in the apoptosis pathway and both are targeted by miR-7-5p; *CDKN1A* and *SMAD3* are transcripts encoding for proteins involved in the cell cycle-related pathway and both are targeted by miR-145-5p. Taken together, these observations indicate miRNAs originated from different families are targeting at the same transcript or a miRNA may cross targeting different transcripts encoding for proteins regulating the same carcinogenesis and metastatic processes.

4.5 Co-expression of miRNA-5p and -3p Pairs in Colon Cancer Cells

One of the focuses of this study is co-expression and regulation of the miRNA-5p and -3p pairs in colon cancer cells. Amongst the 128 differentially expressed miRNAs, a total of 19 pairs of miRNA-5p and -3p (38 miRNAs) were co-expressed in colon cancer cells with $\log_2$(fold change) > 1.5 or < -1.5 (Table 4.5). Besides, 14 miRNA pairs (73.7%) showed statistically significant fold changes ($p < 0.05$) in both miRNA-5p and -3p species; the remaining five pairs were also significantly dysregulated in at least one species of the miRNA pair. The fold changes observed in the cancer cells relative to the cells of normal tissues ranged from significant up-regulation of miR-21-5p by 9.13 ±

0.39 fold to down-regulation of miR-574-3p by -3.94 ± 0.21 fold (Table 4.5). Out of the 19 miRNA pairs, 14 pairs (73.7%) were co-expressed and co-up-regulated and three pairs (15.8%) were co-expressed and co-down-regulated. Only two miRNA pairs (10.5%), let-7d and miR-200b, were inversely regulated in colon cancer cells, i.e. in colon cancer cells, one miRNA species of the miRNA pair was up-regulated and another miRNA species was down-regulated. The data reveal frequent co-expression of miRNA-5p and -3p species in colon cancer cells and the majority of them (17 pairs, 89.5%) were similarly co-up or co-down-regulated, suggesting concerted dysregulation and co-biosynthesis of the miRNA sister pairs in colon cancer cells.

Table 4.5: Co-expression of miRNA-5p and -3p pairs in colon cancer cells relative to normal (n = 19)

miRNA Family[1]	miRNA-5p[1]	log_2(fold change) (Mean ± SD)	miRNA-3p[1]	log_2(fold change) (Mean ± SD)
miRNA-5p/3p co-expression up-regulated (n = 14)				
let-7	let-7g-5p	2.97 ± 0.49**	let-7g-3p	2.12 ± 0.13*
let-7	let-7i-5p	2.81 ± 1.27*	let-7i-3p	1.67 ± 0.45*
mir-7	miR-7-5p	6.44 ± 1.14*	miR-7-1-3p	3.83 ± 0.66**
mir-8	miR-141-5p	3.85 ± 0.76**	miR-141-3p	4.50 ± 1.08**
mir-8	miR-200a-5p	5.17 ± 0.65**	miR-200a-3p	8.10 ± 1.58**
mir-17	miR-17-5p	6.68 ± 0.69**	miR-17-3p	4.12 ± 0.59**
mir-17	miR-18a-5p	3.94 ± 0.91**	miR-18a-3p	3.18 ± 0.77**
mir-17	miR-20a-5p	7.19 ± 0.78**	miR-20a-3p	1.58 ± 0.98
mir-21	miR-21-5p	9.13 ± 0.39**	miR-21-3p	5.99 ± 0.85**
mir-22	miR-22-5p	1.98 ± 0.78	miR-22-3p	3.22 ± 0.76**
mir-27	miR-27a-5p	2.94 ± 1.21**	miR-27a-3p	4.78 ± 0.62**
mir-28	miR-151a-5p	2.65 ± 0.67*	miR-151a-3p	5.39 ± 1.20**
mir-29	miR-29b-1-5p	2.93 ± 0.84**	miR-29b-3p	7.62 ± 0.32**
mir-31	miR-31-5p	8.88 ± 1.73**	miR-31-3p	2.08 ± 0.76**
miRNA-5p/3p co-expression down-regulated (n = 3)				
mir-199	miR-199a-5p	-3.84 ± 2.16**	miR-199a-3p	-3.23 ± 1.55**
mir-378	miR-378a-5p	-2.60 ± 0.008**	miR-378a-3p	-1.04 ± 0.27
mir-574	miR-574-5p	-2.90 ± 0.44*	miR-574-3p	-3.94 ± 0.21**
miRNA-5p/3p inversed regulated (n = 2)				
let-7	let-7d-5p	2.29 ± 1.01	let-7d-3p	-2.78 ± 0.79**
mir-8	miR-200b-5p	-1.67 ± 0.39	miR-200b-3p	3.15 ± 0.25**

[1]Based on miRBase release 19. A full list of alternative or previous miRNA names is provided in Supplementary Table 1 in Appendix A. *$p < 0.05$ and **$p < 0.01$.

4.6 Experimental Validation of Co-expression of miRNA-5p and -3p Pairs

To validate co-expression of miRNA-5p and 3p pairs, one miRNA pair was randomly selected from each co-expression pattern group in Table 4.5. The three randomly selected miRNA pairs were subjected to experimental validation using standard miRNA real-time PCR. At the same time, the validity and consistency of the profiling data were also tested. In real-time PCR validation assay (Figure 4.4 and Table 4.6), miR-20a-5p and 3p were significantly up-regulated in colon cancer cells by 7.74 ± 1.17 and 4.23 ± 1.01 fold, respectively; let-7d-5p was significantly up-regulated by 4.08 ± 0.87 fold and 3p was significantly down-regulated by 2.96 ± 0.33 fold in colon cancer cells; miR-378a-5p was down-regulated by 1.13 ± 0.87 fold, showing that real-time PCR data were consistent with the WaferGen SmartChip MicroRNA PCR array profiling data, thus, supporting the validity of the profiling data in this study. The only inconsistent data was found in the 3p species of the miR-378a pair as it was up-regulated in real-time PCR validation and down-regulated in WaferGen PCR array; however, the $\log_2$(fold change) data obtained from the SmartChip MicroRNA array and the real-time PCR validation experiments showed that miR-378a-3p was not significantly regulated in colon cancer cells.

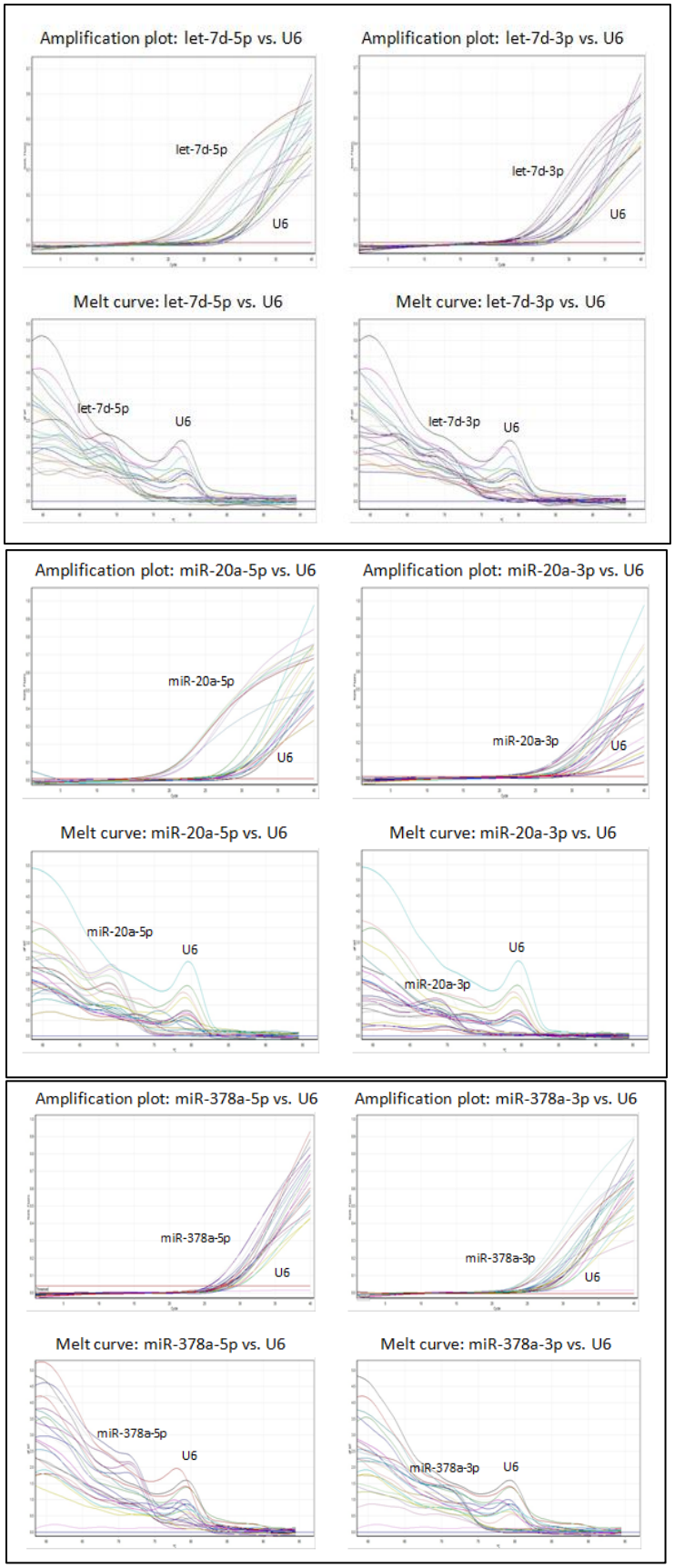


Figure 4.4: Amplification plot and melt curve for miRNA real-time PCR analysis on selected miRNA-5p and -3p pairs. U6 was used as endogenous normalising control.

Table 4.6: Experimental validation of dysregulated miRNA-5p and -3p pairs in colon cancer cells relative to normal colon cells

miRNA	WG miRNA panel[1]	Real-time PCR validation	Expression mode
	$\log_2$(fold change) (Mean ± SD)	$\log_2$(fold change) (Mean ± SD)	
miR-20a-5p	7.19 ± 0.78**	7.74 ± 1.17**	Up-regulated
miR-20a-3p	1.58 ± 0.98	4.23 ± 1.01**	Up-regulated
miR-378a-5p	-2.60 ± 0.003**	-1.13 ± 0.87	Down-regulated
miR-378a-3p	-1.04 ± 0.27	1.06 ± 1.01	Not significant
let-7d-5p	2.29 ± 1.01	4.08 ± 0.87**	Up-regulated
let-7d-3p	-2.78 ± 0.79**	-2.96 ± 0.33**	Down-regulated

[1]WaferGen Biosystems SmartChip Human MicroRNA Panel v2. *$p < 0.05$ and **$p < 0.01$.

4.7 Target Transcripts and Putative Biological Functions of miRNA-5p and -3p Pairs

To study putative biological functions of target transcripts of the miRNA-5p and -3p pairs and to further elucidate potential cancer-related regulatory pathways that may be regulated by these miRNAs, transcripts and biological functions of the paired miRNAs are determined (Table 4.7). This study was performed using miRNA target prediction algorithms including miRBase and TargetScanHuman; gene ontology analysis tools including NCBI gene-gene ontology database and KEGG pathway database, as described in Section 3.6. For a full list of the target transcripts and functions of the 19 co-expressed miRNA pairs in colon cancer cells, see Supplementary Table 2 in Appendix B.

Table 4.7: Cancer-related phenotypes/biological functions of selected target transcripts of miRNA-5p and -3p pairs (n = 19 pairs)

miRNA family	Chromosomal location	miRNA	Expression status	Predicted/Validated targets[1]	Function/Phenotype(s) affected[1]
let-7	9q22.32	let-7d-5p	Up-regulated	*IGF1R*[#], *THBS1*[#], *HMGA2*	Angiogenesis, apoptosis, cell cycle, EMT, p53 signalling
		let-7d-3p	Down-regulated	*KRAS*, *PRKACB*	Apoptosis, MAPK signalling pathway, G-protein regulatory
let-7	3p21.1	let-7g-5p	Up-regulated	*IGF1R*[#], *MYC*[#]	Angiogenesis, apoptosis, MAPK signalling pathway
		let-7g-3p	Up-regulated	*ZEB1*, *ITGA1*, *NLK*	EMT, Angiogenesis, MAPK signalling pathway
let-7	12q14.1	let-7i-5p	Up-regulated	*IL13*[#], *TLR4*[#]	Cell-cell signalling, Toll-like receptor signalling, inflammatory response
		let-7i-3p	Up-regulated	*IGFBP3*, *STAG2*	p53 signalling pathway, cell cycle
mir-7	9q21.32	miR-7-5p	Up-regulated	*IGF1R*[#], *EGFR*[#]	Angiogenesis, apoptosis, MAPK signalling pathway
		miR-7-1-3p	Up-regulated	*IGF1R*, *JUN*	Angiogenesis, apoptosis, MAPK signalling pathway
mir-8/200	12p13.31	miR-141-5p	Up-regulated	*ATM*, *CLDN1*	Apoptosis, cell cycle, p53 signalling pathway, cell adhesion molecules (CAMs)
		miR-141-3p	Up-regulated	*ZEB1/2*[#], *MAPK14*	EMT, angiogenesis, MAPK signalling pathway
mir-8/200	1p36.33	miR-200a-5p	Up-regulated	*FGF4*, *ZEB2*	Apoptosis, MAPK signalling pathway, EMT
		miR-200a-3p	Up-regulated	*ZEB1/2*[#], *SELE*	EMT, cell adhesion molecules (CAMs)
mir-8/200	1p36.33	miR-200b-5p	Down-regulated	*PRDM6*, *ZEB2*	Chromatin modification, EMT
		miR-200b-3p	Up-regulated	*ZEB1*[#], *MLH1*	EMT, mismatch repair

Table 4.7 continued:

miRNA family	Chromosomal location	miRNA	Expression status	Predicted/Validated targets[1]	Function/Phenotype(s) affected[1]
mir-17	13q31.3	miR-17-5p	Up-regulated	*E2Fs*[#], *THBS1*[#], *TGFBR2*[#], *PTEN*[#]	Apoptosis, cell cycle, angiogenesis, EMT, p53 signalling pathway
		miR-17-3p	Up-regulated	*CD44, MYB*	Angiogenesis, inflammation, cancer
mir-17	13q31.3	miR-18a-5p	Up-regulated	*THBS1*[#], *ATM, TGFBR2*[#], *PTEN*[#]	Apoptosis, cell cycle, EMT, p53 signalling pathway
		miR-18a-3p	Up-regulated	*CASP7, KRAS*[#]	Apoptosis, MAPK signalling pathway
mir-17	13q31.3	miR-20a-5p	Up-regulated	*E2Fs*[#], *THBS1*[#], *TGFBR2*[#], *PTEN*[#]	Apoptosis, cell cycle, angiogenesis, EMT, p53 signalling pathway
		miR-20a-3p	Up-regulated	*KRAS, PCNA*	Apoptosis, MAPK signalling pathway, cell cycle
mir-21	17q23.1	miR-21-5p	Up-regulated	*TGFBR2*[#], *PTEN*[#]	EMT, apoptosis, p53 signalling pathway
		miR-21-3p	Up-regulated	*CASP7, TGFB2*	Apoptosis, EMT
mir-22	17p13.3	miR-22-5p	Up-regulated	*NEXN, MAP3K2*	Cell migration, MAPK signalling pathway
		miR-22-3p	Up-regulated	*NR3C1, BMP7*[#]	Apoptosis, cell cycle, EMT
mir-27	19p13.13	miR-27a-5p	Up-regulated	*ATM, PDGFA*	Cell cycle, apoptosis, angiogenesis
		miR-27a-3p	Up-regulated	*FOXO1*[#], *EGFR*[#], *ATM*	Apoptosis, MAPK signalling pathway
mir-28	8q24.3	miR-151a-5p	Up-regulated	*ARHGDIA, CYP2E1*	Apoptosis, drug metabolism – cytochrome P450/metabolic pathways
		miR-151a-3p	Up-regulated	*THBS1, ZEB1*	Angiogenesis, cell cycle, EMT, p53 signalling pathway

Table 4.7 continued:

miRNA family	Chromosomal location	miRNA	Expression status	Predicted/Validated targets[1]	Function/Phenotype(s) affected[1]
mir-29	7q32.3	miR-29b-1-5p	Up-regulated	*PRMT8, TCF7L2*	DNA methylation, apoptosis, cell cycle, EMT
		miR-29b-3p	Up-regulated	*DNMT3A/B, PTEN*	DNA methylation, apoptosis, p53 signalling pathway
mir-31	9p21.3	miR-31-5p	Up-regulated	*TP53, RHOA*[#]	Angiogenesis, apoptosis, cell cycle, p53, MAPK signalling pathway, inflammation
		miR-31-3p	Up-regulated	*ACVR1, VDAC1*	Apoptosis, inflammation
mir-199	19p13.2; 1q24.3	miR-199a-5p	Down-regulated	*IKBKB*[#], *CAV1*[#], *GSK3B*	Apoptosis, MAPK signalling pathway, EMT
		miR-199a-3p	Down-regulated	*MET*[#], *MAPK8*[#]	Angiogenesis, apoptosis, MAPK signalling pathway
mir-378	5q33.1	miR-378a-5p	Down-regulated	*DCC, GABPA*	Apoptosis
		miR-378a-3p	Down-regulated	*MSH3, SUFU*[#]	DNA repair, promote angiogenesis and tumour growth
mir-574	4p14	miR-574-5p	Down-regulated	*FOXN3*[#], *BMP4*	DNA damage response, angiogenesis, inflammation
		miR-574-3p	Down-regulated	*CHEK1, CARM1*	Cell cycle, DNA repair, p53 signalling, histone methylation

A full list of predicated/validated target transcripts and putative functions of the 19 co-expressed miRNA pairs is presented in Supplementary Table 2 in Appendix B. Data in each group are depicted in the order of miRNA family name. Hash (#) indicates validated target. [1]Data derived from the miRNA target prediction algorithm (as described in Materials and Methods Section 3.6), NCBI Gene Ontology Annotation Database (UniProt-GOA) and KEGG Pathway databases.

Data showed that most of target transcripts of these 19 pairs of miRNA-5p and -3p (38 miRNAs), either predicted or validated target transcripts, are involved in specific cancer-related regulatory pathways: 27 miRNAs (71.1%) are apoptosis-related, 21 miRNAs (55.3%) are angiogenesis-related, 19 miRNAs (50%) are cell cycle-related, 16 miRNAs (42.1%) are EMT-related, 16 miRNAs (42.1%) are p53 signalling pathway-related, and 16 miRNAs (42.1%) are MAPK signalling pathway-related (Tables 4.7 – 4.8). The data indicate that the identified miRNA-5p and -3p pairs are also actively engaged in multiple cancer-related pathways and involved in regulation of various key cellular processes in carcinogenesis. The predicted or validated target transcripts extracted from databases are mapped onto the EMT pathway, p53 signalling pathway-related apoptosis and cell cycle arrest pathways that are available from the KEGG Pathway Database (Figures 4.4 – 4.6).

In target prediction analysis, the data revealed that, target of only one species of the miRNA pair was validated in most cases (Table 4.7). The validated miRNAs, either miRNA-5p or -3p, are most likely the guiding strand miRNA species that are maintained in significantly higher concentration in the cell than the passenger strand miRNA species that is usually expressed in much lower level that has still yet to be experimentally validated.

Table 4.8: Putative target transcripts of miRNA-5p and -3p pairs[1] involved in cancer-related regulatory pathways

Process	Total number of miRNAs	5p/3p pairing of miRNA	Non-pairing miRNA
EMT	16 (42.1%)	6 (3 pairs)	10
p53 signalling pathway	16 (42.1%)	4 (2 pairs)	12
Apoptosis	27 (71.1%)	22 (11 pairs)	5
Cell cycle	19 (50%)	8 (4 pairs)	11
Angiogenesis	21 (55.3%)	12 (6 pairs)	9
MAPK signalling pathway	16 (42.1%)	6 (3 pairs)	10

Data shown above are the summary of Table 4.7; [1]n = 19 pairs.

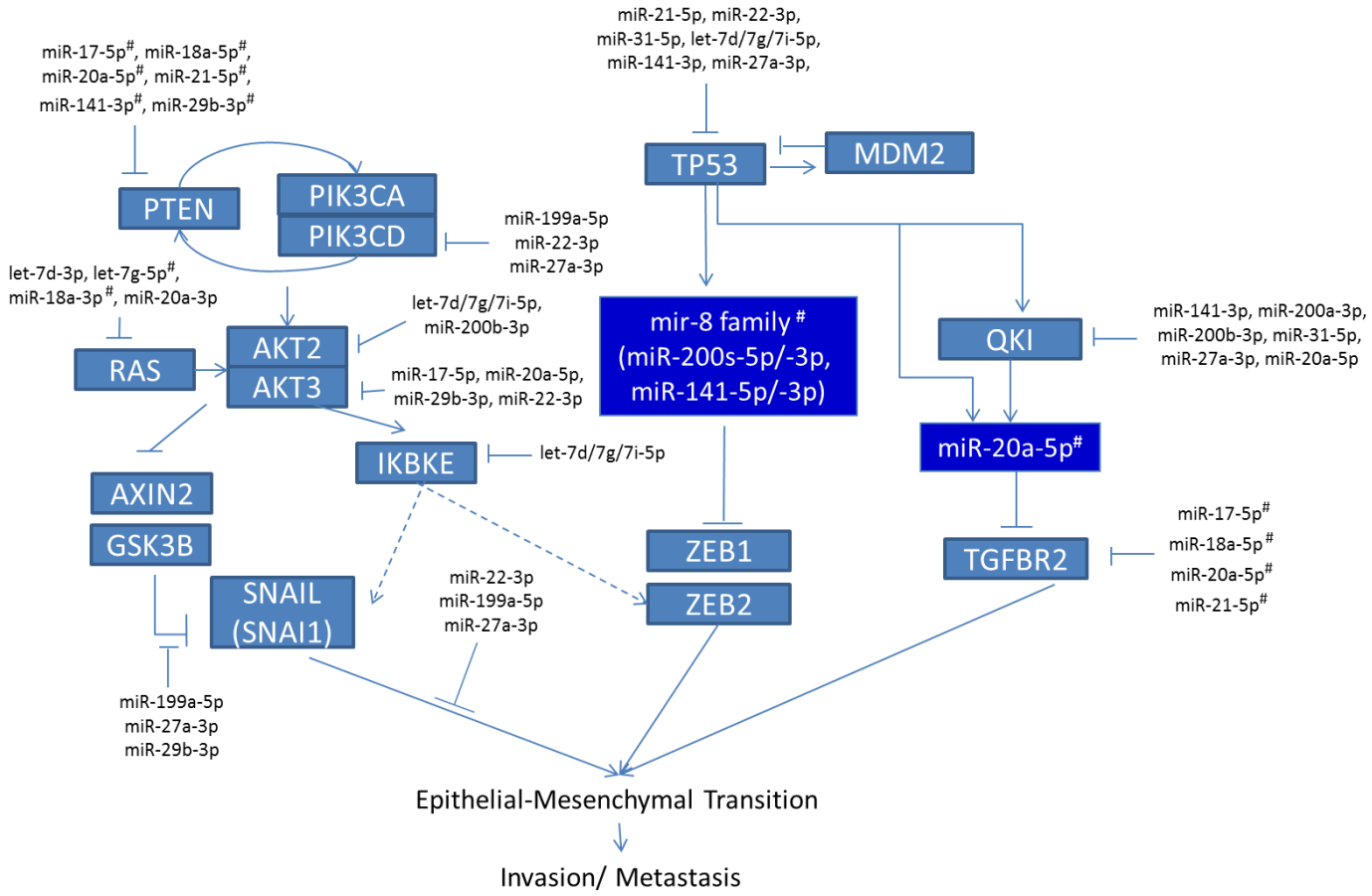


Figure 4.5: miRNAs targeting transcripts involved in Epithelial-Mesenchymal Transition (EMT) pathway. Hash (#) denotes validated miRNA target transcripts.

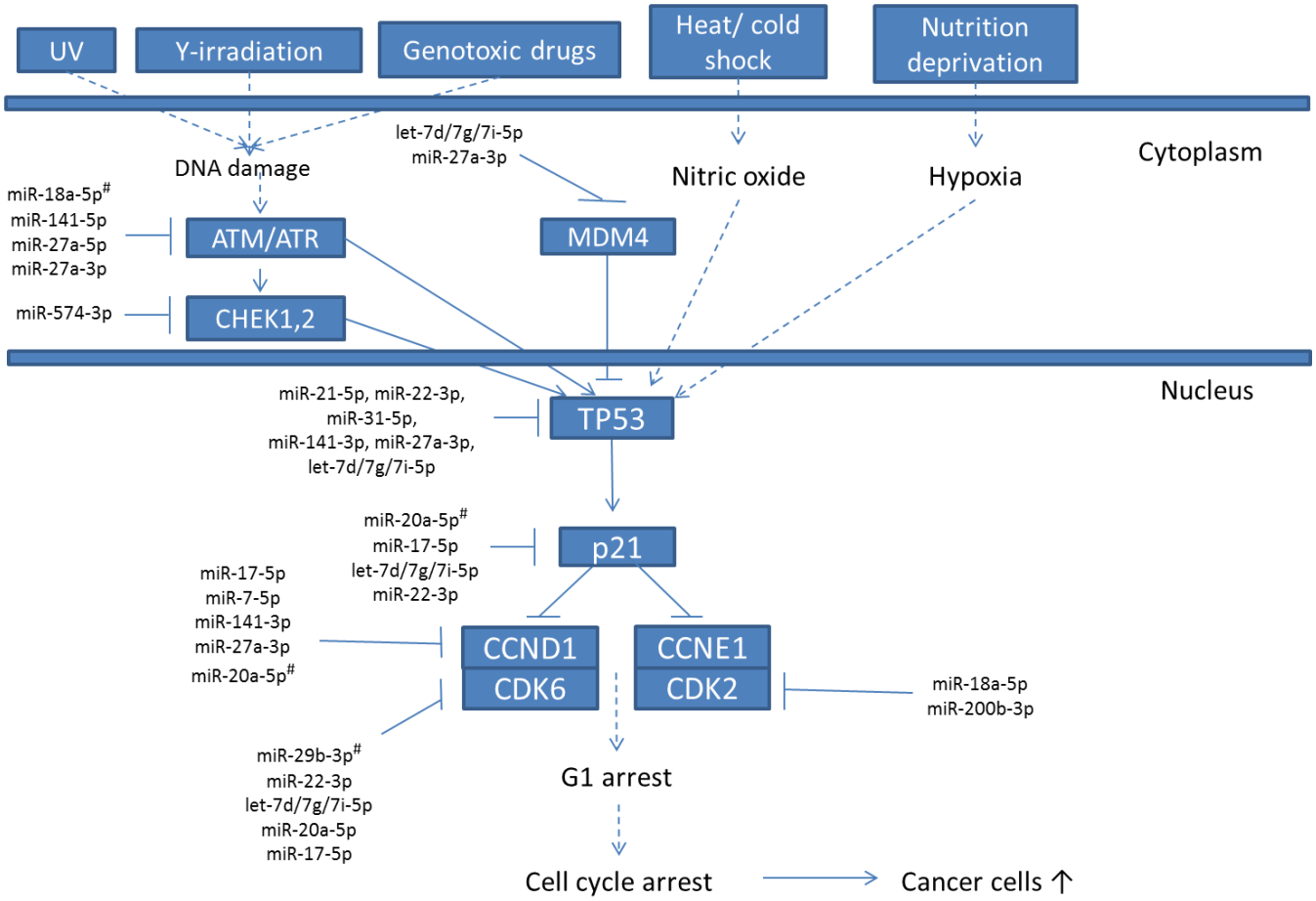


Figure 4.6: miRNAs targeting transcripts involved in p53 signalling pathway-related cell cycle arrest (KEGG Pathway Database). Hash (#) denotes validated miRNA target transcripts.

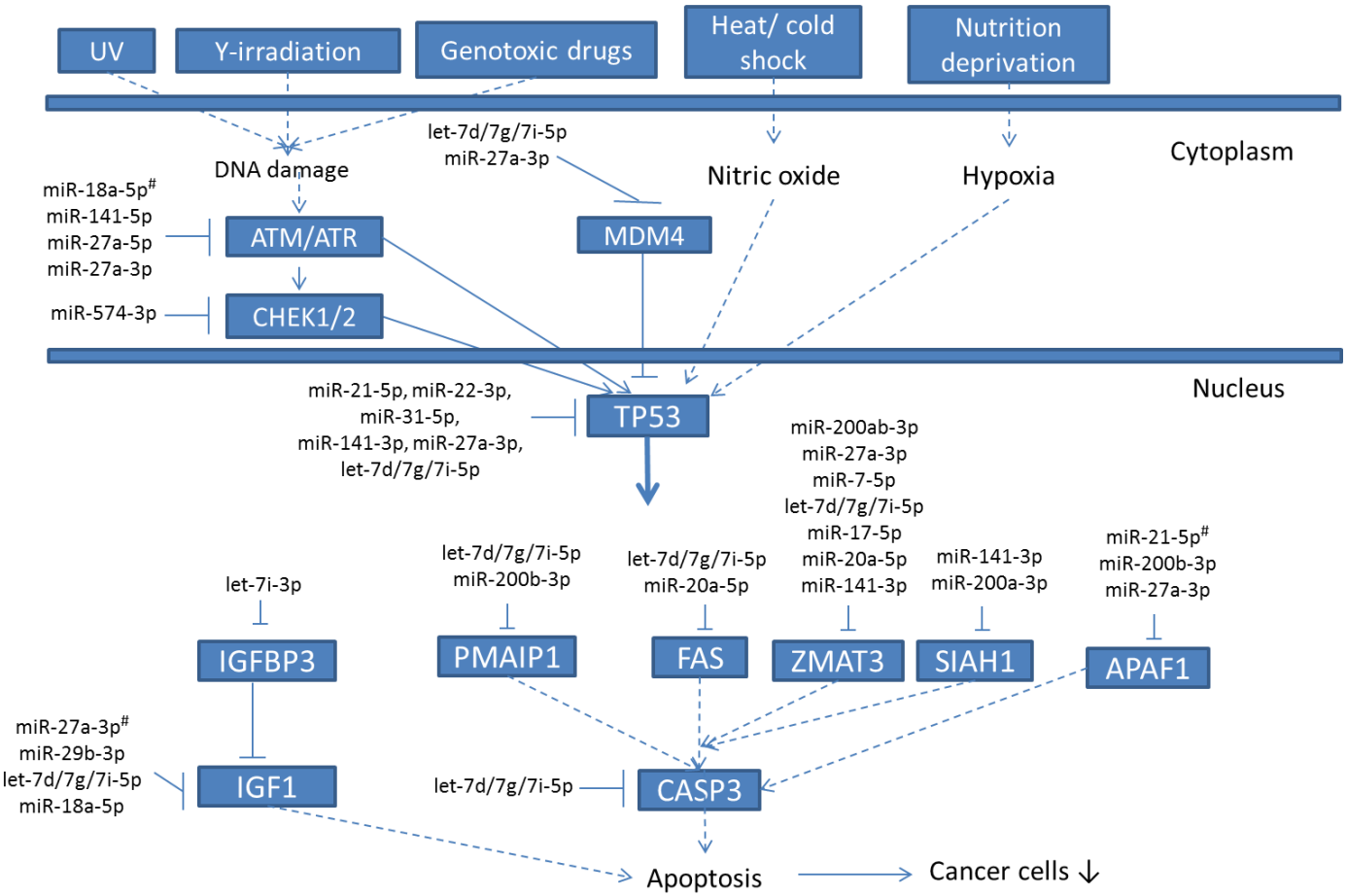


Figure 4.7: miRNAs targeting transcripts involved in p53 signalling pathway-related apoptosis (KEGG Pathway Database). Hash (#) denotes validated miRNA target transcripts.

Members from the same miRNA family are predicted to possess similar functions and regulate specific pathways as they are found to be targeted on similar gene transcript set (Table 4.7, Figure 4.5 - 4.7). As shown in Table 4.7, both *TGFBR2* and *PTEN* transcripts are involved in the apoptosis pathway and both are the target transcripts for miR-17-5p, miR-18a-5p and miR-20a-5p from the mir-17 family. In addition, these mir-17 family members are also predicted to be targeted on *THBS1* transcript, which encodes for protein involved in multiple processes including angiogenesis, cell cycle and p53 signalling pathway (Table 4.7).

On the other hand, multiple miRNAs of different families are also revealed to target the same target transcript and possess similar functions. *ATM* transcript is targeted by miR-18a-5p, miR-141-5p and miR-27a of different miRNA families (Table 4.7); *PTEN* is targeted by the mir-17 family, miR-21-5p, miR-141-3p and miR-29b-3p in the EMT pathway (Figure 4.5). Besides, *IGF1R* and *MYC* transcripts are cross-targeted by let-7g-5p in the regulation of the angiogenesis process in cancer (Table 4.7). Taken together, our data indicate that multiple miRNAs may be targeting at the same transcript, or a miRNA may cross-targeting different transcripts encoding for different proteins in the regulation of the carcinogenesis and metastasis processes.

Interestingly, miRNA 5p/3p pairs are revealed to regulate the same biological process, i.e. both the miRNA-5p and -3p which are derived from the

same precursor but have different sequences may still exert similar biological functions, by targeting on the same or different transcript. There are eleven pairs of miRNA-5p and -3p that appear to be involved in the apoptosis pathway, six pairs in the angiogenesis process and four pairs in the cell cycle (Table 4.8). In the target-transcript prediction analysis, miR-7-5p and miR-7-1-3p derived from the same precursor, either mir-7-1 or mir-7-2 or mir-7-3, are found to target the *IGF1R* transcript (Table 4.7), miR-27a-5p and miR-27a-3p derived from the same precursor mir-27a are targeting the *ATM* transcript (Table 4.7, Figures 4.5 - 4.6). Besides, analysis also reveals differential miRNA targeting derived from the same precursor. For example, miR-141-3p is predicted to target the *SIAH1* and *ZMAT3* transcripts in the apoptosis pathway, while miR-141-5p is predicted to target *ATM* also in the same pathway.

4.8 *IGF1R* and *KRAS* are Putative Targets for let-7d-5p and -3p Pair miRNAs, respectively

Throughout Figure 4.5 – 4.7, the differentially expressed let-7 family members, let-7d, let-7g and let-7i (particularly the -5p species) are shown to target on multiple transcripts involved in the apoptosis, cell cycle arrest and EMT pathways, suggests that the let-7 family members, let-7d/7g/7i-5p are biologically significant in apoptosis, cell cycle arrest and EMT in the carcinogenesis processes. Therefore, one pair of the let-7 family members, let-7d-5p and 3p was chosen based on their novelty in reported colon cancer-

related studies. Besides, it is important to further validate the regulation modes of let-7d-5p and -3p pair that was surprisingly found to be inversely regulated in colon cancer cells, i.e. let-7d-5p was up-regulated and let-7d-3p was down-regulated (Tables 4.5 - 4.6). Web-based bioinformatics algorithms as described in Materials and Methods Section 3.6 were used to predict miRNA target transcripts. Data reveal that let-7d-5p is predicted to target the 7088-bp 3'-untranslated region (3'-UTR) of insulin-like growth factor 1 receptor (*IGF1R*) mRNA transcript at three different sites (Figure 4.8): nucleotide (nt) 99-105 (complementary 6-mer seed sequence matching), nt 2619-2626 (complementary 8-mer seed sequence matching) and nt 6661-6667 (complementary 8-mer seed sequence matching). Similarly, let-7d-3p is predicted to target the 4564-bp 3'-UTR of the mRNA of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) at nt 4277-4282 with a complementary 6-mer seed matching site (Figure 4.9). The putative miRNA target binding sites of 3'-UTR of the *IGF1R* and *KRAS* transcripts are highly conserved across mammalian species (Figures 4.8 - 4.9).

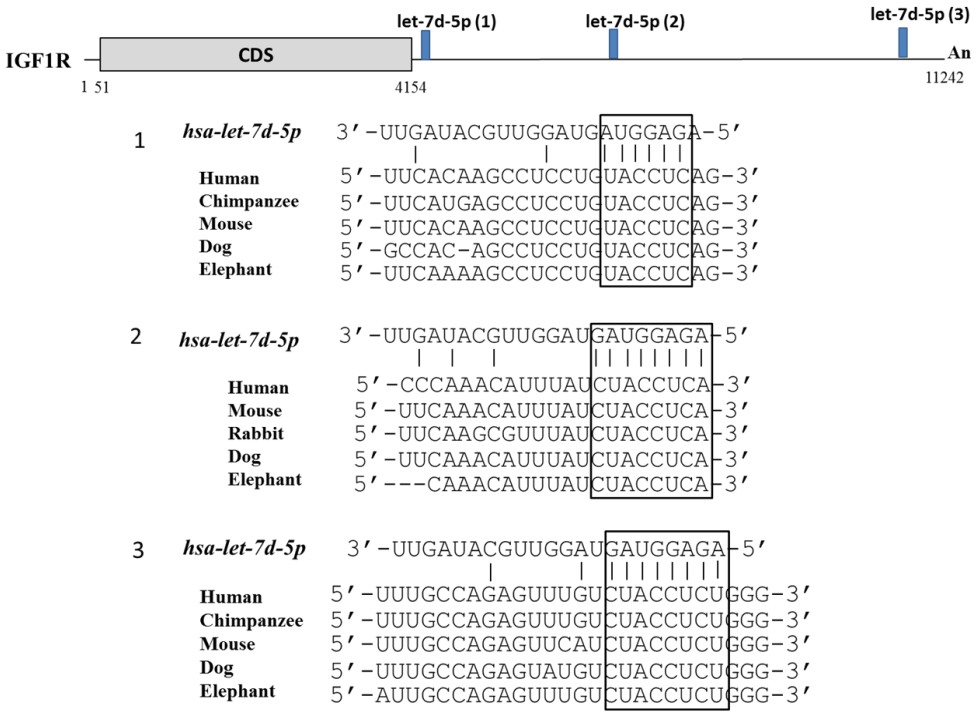


Figure 4.8: Insulin-like growth factor 1 receptor (*IGF1R*) is a putative target for let-7d-5p at three sites.

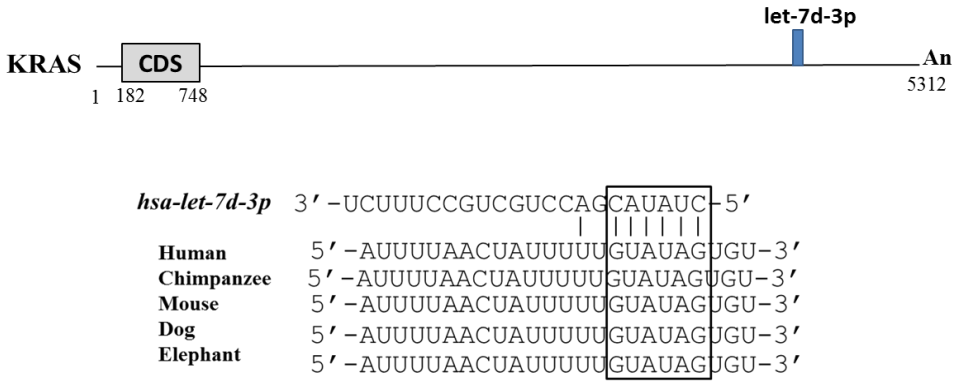


Figure 4.9: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) is a putative target for let-7d-3p.

To investigate miRNA post-transcriptional regulation of both the *IGF1R* and *KRAS* transcripts, mRNA and protein levels of both transcripts were measured by real-time PCR and western blot analysis. The concentration of protein lysates prepared from colon cancer cells (Supplementary Figure 1 and Table 3) used for western blot analysis was determined. In real-time PCR analysis, data revealed the comparable *IGF1R* transcript expression levels among the four cell lines, the average fold change was determined. The data showed that *IGF1R* transcript was significantly down-regulated by 3.80 ± 1.63 fold ($p < 0.01$) in the four colon cancer cells relative to normal cells (Figure 4.10 and Table 4.9). In western blot analysis, the IGF1R α-subunit 130 kDa protein and β-subunit 97 kDa protein (cleavage product of IGF1R precursor) showed very faint bands in colon cancer cells, both subunits were down-regulated by 0.33 ± 0.23 fold ($p < 0.01$) and 0.53 ± 0.37 fold ($p < 0.01$) in colon cancer cells relative to normal (Figure 4.11 and Table 4.10); the IGF1R protein was found to be expressed at similar levels among the cell lines. Likewise, *KRAS* transcript was significantly down-regulated in colon cancer cells relative to normal cells by 2.12 ± 1.05 ($p < 0.05$) at the mRNA level (Figure 4.10 and Table 4.9). The KRAS 21 kDa protein was, on the other hand, significantly up-regulated by 4.85 ± 1.39 fold ($p < 0.01$) in colon cancer cells relative to the normal (Figure 4.11 and Table 4.10). In addition, the KRAS protein was found to be expressed at similar levels among the cell lines. The additional upper band detected in western blotting analysis of KRAS in normal colon is KRAS isoform, an unprocessed form of KRAS, whereas the KRAS 21 kDa protein detected in colon cancer cells is a processed form of KRAS – phosphorylated KRAS (Rebollo et al., 1999; Ahearn et al., 2012).

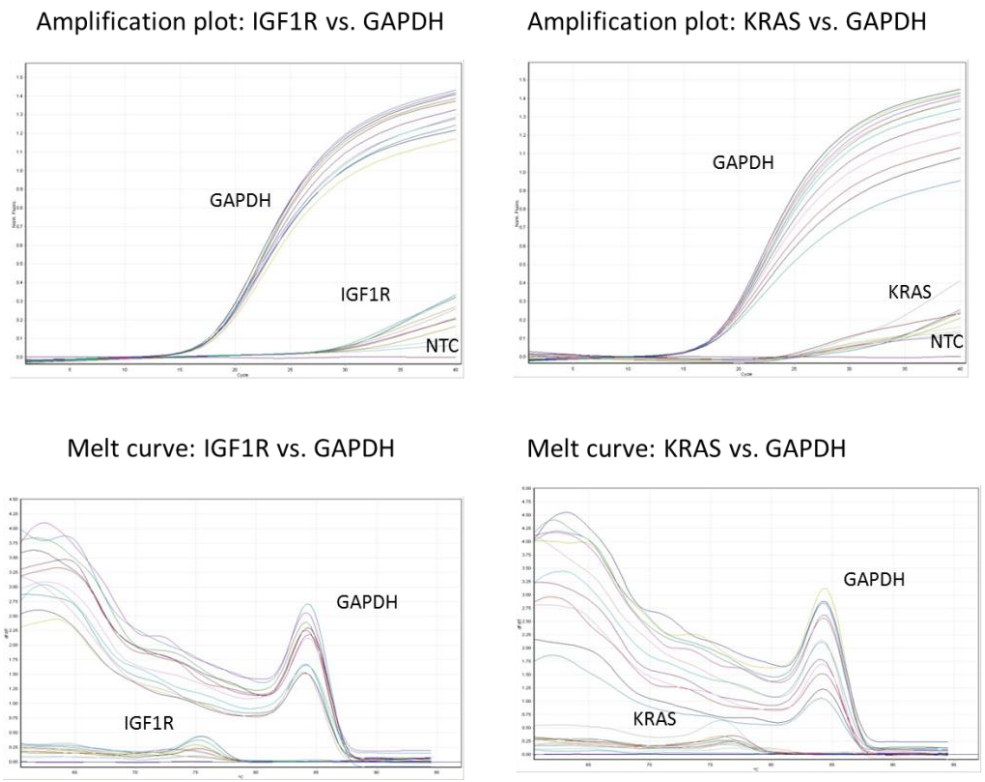


Figure 4.10: Amplification plot and melt curve for real-time PCR analysis on *IGF1R* and *KRAS* transcripts. *GAPDH* was used as endogenous normalising control. NTC: Non-template control.

Table 4.9: Expression levels of let-7d-5p and -3p pair and targeted mRNA transcripts in colon cancer cells (n = 3)

miRNA	Cell lines	miRNA expression log_2(fold change)	Target transcript	mRNA expression log_2(fold change)
		WG panel		**Real-time PCR**
let-7d-5p	HCT-15	1.63	*IGF1R*	-4.83 ± 0.71
	HT-29	3.49		-2.03 ± 0.63
	SK-CO-1	1.29		-5.49 ± 1.29
	WiDr	2.74		-2.86 ± 0.47
Average of the four cell lines		2.29 ± 1.01		-3.80 ± 1.63**
let-7d-3p	HCT-15	-3.91	*KRAS*	-3.44 ± 0.25
	HT-29	-2.10		-1.36 ± 0.31
	SK-CO-1	-2.41		-2.47 ± 1.85
	WiDr	-2.68		-1.19 ± 0.83
Average of the four cell lines		-2.78 ± 0.79**		-2.12 ± 1.05*

HCT-15, HT-29, SK-CO-1 and WiDr colon cancer cells were used in the analysis; Data shown are mean ± SD of three independent experiments; *$p < 0.05$, **$p < 0.01$.

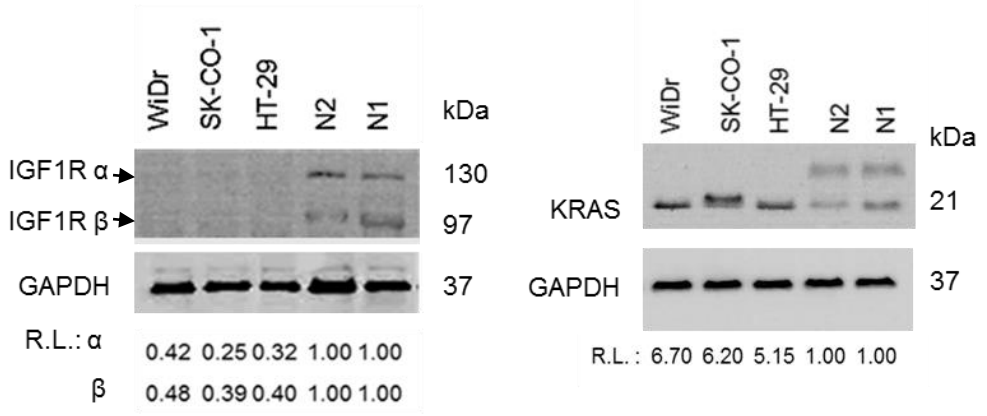


Figure 4.11: Western blotting analysis of let-7d-5p and -3p target proteins IGF1R and KRAS in colon cancer cells. Band densitometric analysis was performed using UVP VisionWorks®LS Image Acquisition and Analysis Software. Based on three independent experiments, average of protein expression levels relative to normal is showed in Table 4.10. Upper band in western blotting analysis of KRAS in N1 and N2, unprocessed form of KRAS; KRAS 21 kDa protein, phosphorylated KRAS. N1 and N2 are two commercially available protein lysates from normal colonic tissues derived from two different donors. R.L., protein expression level relative to normal colonic tissue protein lysates.

Table 4.10: Expression levels of let-7d-5p and -3p and targeted proteins in colon cancer cells

miRNA	Cell lines	miRNA expression log_2(fold change)	Target transcript	Protein expression
		WG panel		**Western blot**
let-7d-5p	HT-29	3.49	IGF1R	α : 0.25 ± 0.20 β : 0.51 ± 0.45
	SK-CO-1	1.29		α : 0.22 ± 0.11 β : 0.37 ± 0.12
	WiDr	2.74		α : 0.50 ± 0.30 β : 0.71 ± 0.49
Mean		2.50 ± 1.12		α : 0.33 ± 0.23** β : 0.53 ± 0.37**
let-7d-3p	HT-29	-2.10	KRAS	4.08 ± 1.25
	SK-CO-1	-2.41		4.01 ± 2.11
	WiDr	-2.68		6.47 ± 2.81
Mean		-2.40 ± 0.29**		4.85 ± 1.39**

HT-29, SK-CO-1 and WiDr were cell lines included in the analysis; *$p < 0.05$, **$p < 0.01$.

The putative targets of let-7d pair, *IGF1R* and *KRAS* were experimentally validated by alteration of the endogenous miRNA levels. The effects of changes on the target transcript mRNA and protein levels due to altered miRNAs were measured. The mRNA expression levels of *IGF1R* and *KRAS* were most down-regulated in HCT-15 and SK-CO-1 cells – the metastatic cell types, however, let-7d-5p was not significantly expressed in SK-CO-1 cells (Table 4.9). Hence, HCT-15 cells were used in the miRNA knockdown or overexpression assay.

let-7d-5p level was knocked-down by transient transfection of HCT-15 cells with a specific let-7d-5p miRNA inhibitor (Figure 4.12). Consequently, *IGF1R* mRNA was significantly up-regulated by 2.58 ± 0.59 fold ($p < 0.05$) (Table 4.11 and Figure 4.12) and there was only insignificant effects on *IGF1R* mRNA level in the cells transfected with a miRNA inhibitor negative control which was composed of a random miRNA sequence. When the let-7d-5p level was knocked-down, IGF1R protein was significantly up-regulated by 1.64 ± 0.19 fold ($p < 0.01$) relative to mock control (Table 4.11 and Figure 4.12), supporting regulation of IGF1R possibly by let-7d-5p by increasing *IGF1R* mRNA level and, thus, increasing protein synthesis. The let-7d-3p level was overexpressed by transient transfection of HCT-15 cells with a specific let-7d-3p miRNA mimic (Figure 4.13). The increased of miRNA level had led to significant down-regulation of *KRAS* mRNA by 0.30 ± 0.23 fold ($p < 0.05$) (Table 4.11 and Figure 4.13) but there was only insignificant changes on *KRAS* mRNA level in the cells transfected with a miRNA mimic negative

control. The increased miRNA level also led to significant down-regulation of the KRAS protein by 0.52 ± 0.09 fold ($p < 0.01$) relative to mock control (Table 4.11 and Figure 4.13), and hence, supporting let-7d-3p regulation of KRAS. Taken together, data of the miRNA transfection assays support differential targeting of IGF1R and KRAS transcripts by let-7d-5p and -3p, respectively, at both the transcript and protein levels. Future experiments to investigate the mRNA and protein level changes after HCT-15 colon cancer cells treated with let-7d-5p mimic and let-7d-3p inhibitor is essential to further support the findings.

Table 4.11: Expression levels of let-7d-5p and -3p and targeted protein in HCT-15 cells after miRNA inhibitor or mimic transfection

miRNA	Oligo Transfected	miRNA FC[1] (after mimic or inhibitor transfection)	Target transcript	mRNA FC[1]	Protein FC[1]
let-7d-5p	7d-5p inhibitor	0.01	IGF1R	2.58 ± 0.59*	1.64 ± 0.19**
let-7d-3p	7d-3p mimic	477.71	KRAS	0.30 ± 0.23*	0.52 ± 0.09**

[1]FC: Fold change relative to mock control, non-treated cell supplemented with serum-free DMEM (with Lipofectamine RNAiMAX Reagent); *$p < 0.05$, **$p < 0.01$. In this experiment, fold change is used instead of $\log_2$(fold change) because all the fold change values are not exceeding 500.

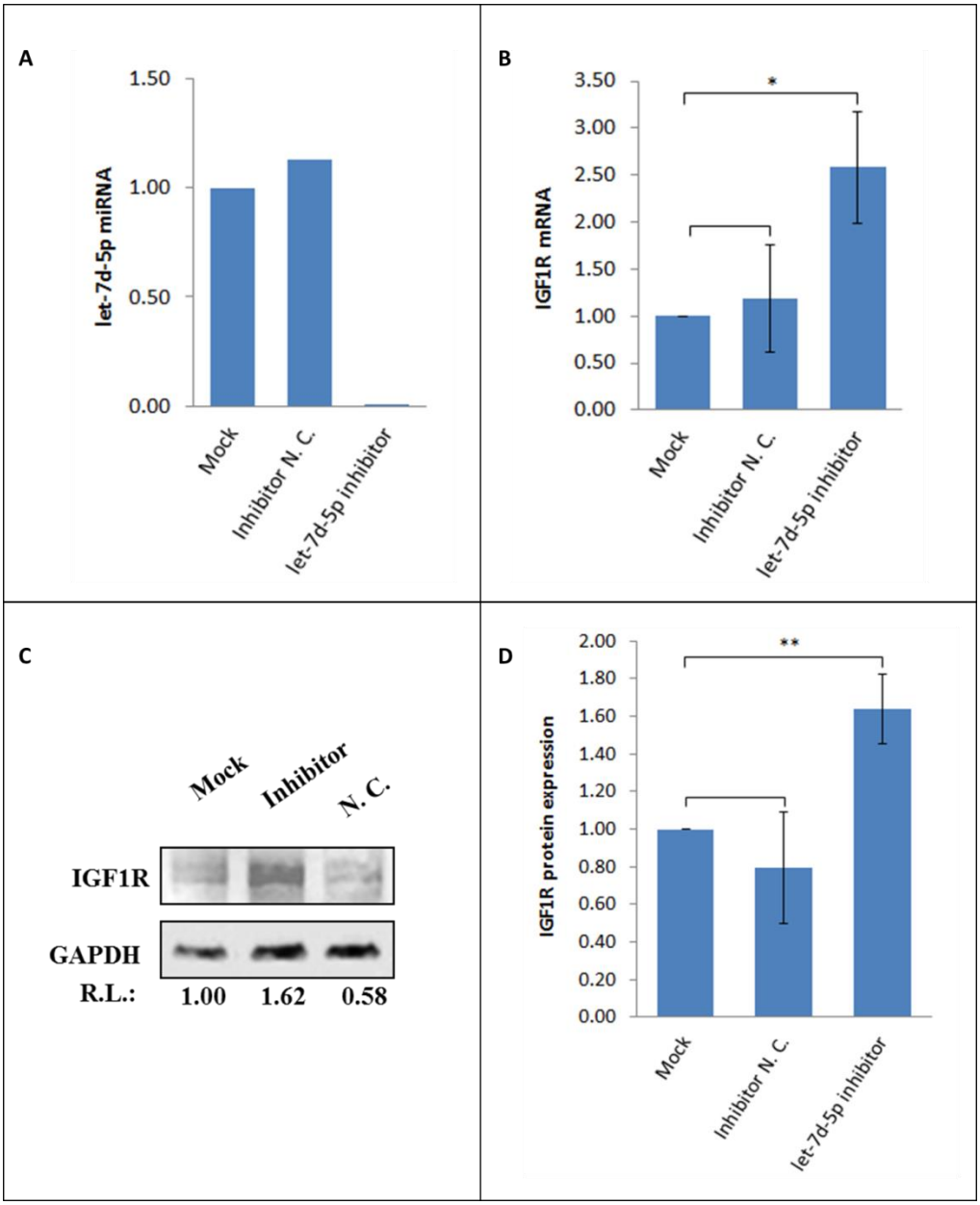


Figure 4.12: IGF1R transcript and protein expression in HCT-15 colon cancer cells treated with the miRNA inhibitor let-7d-5p. A: let-7d-5p miRNA levels in HCT-15 cells after transfection (the miRNA real-time PCR experiment was done one time to check if transfection assay is successful), B: target gene *IGF1R* mRNA levels in HCT-15 cells after transfection, C & D: target protein IGF1R levels in HCT-15 cells after transfection. Each bar in B and D represents fold-change values obtained from three independent experiments. Mock, non-treated cell supplemented with serum-free DMEM (with Lipofectamine RNAiMAX Reagent). N.C., cell treated with miRNA inhibitor negative control; *$p < 0.05$, **$p < 0.01$.

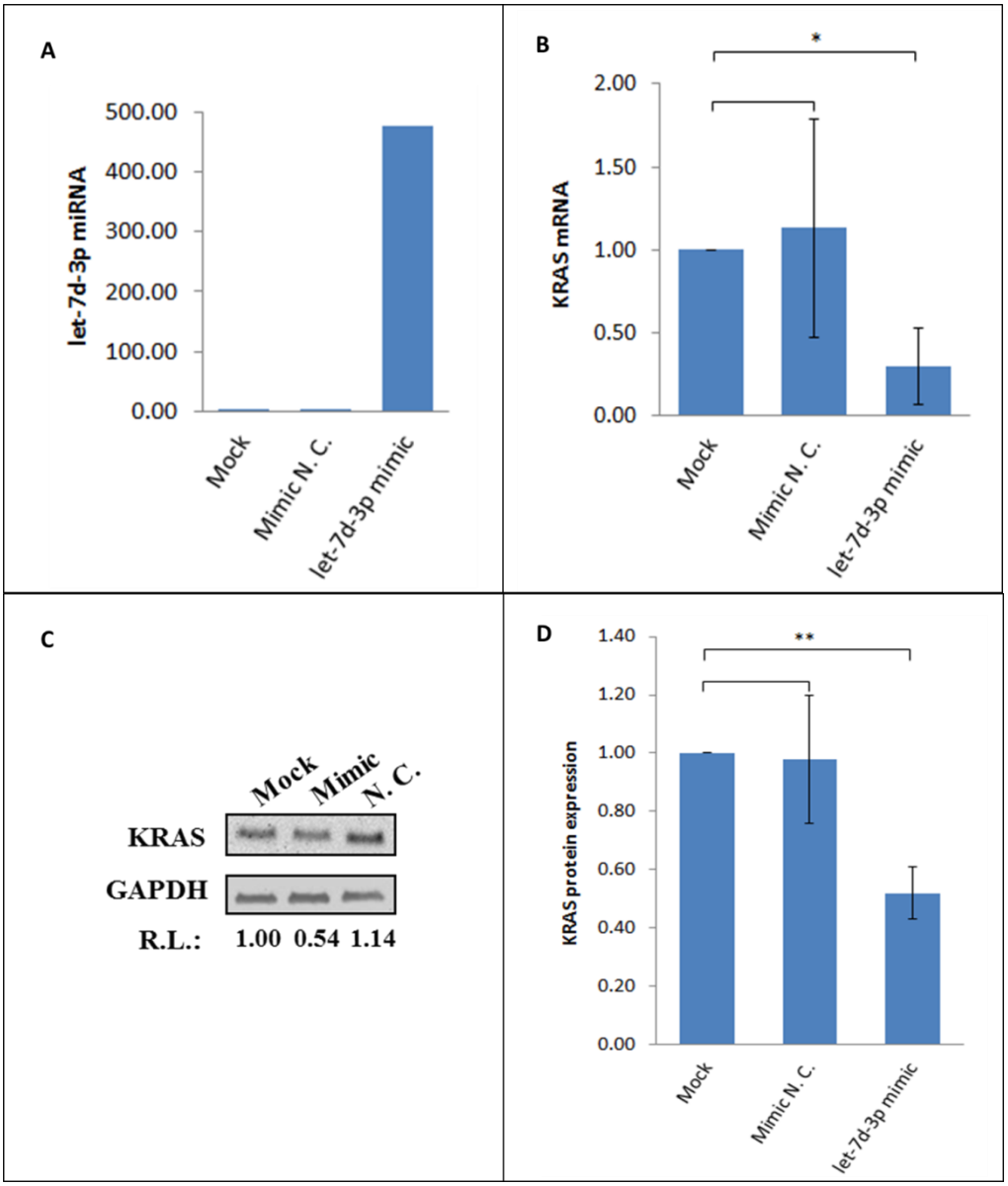


Figure 4.13: KRAS transcript and protein expression in HCT-15 colon cancer cells treated with miRNA mimic let-7d-3p. A: let-7d-3p miRNA levels in HCT-15 cells after transfection (experiment was done one time to check if transfection assay is successful), B: target gene *KRAS* mRNA levels in HCT-15 cells after transfection, C & D: target protein KRAS levels in HCT-15 cells after transfection. Each bar in B and D represents fold-change values obtained from three independent experiments. Mock, non-treated cell supplemented with serum-free DMEM (with Lipofectamine RNAiMAX Reagent); N.C., cell treated with miRNA mimic negative control; *$p < 0.05$, **$p < 0.01$.

CHAPTER 5

DISCUSSION

5.1 miRNA Expression Profiles Reveal Frequent Co-expression of miRNA-5p and -3p Pairs in Colon Cancer Cells

In recent years, miRNA profiling studies in various cancers have been abundantly reported, revealing the involvement of miRNA in colon carcinogenesis (Table 5.1). Echoing previous reports, our data also revealed differential regulation of 128 miRNAs in colon cancer cells, signifying dysregulation of miRNA in colon carcinogenesis.

Hierarchical clustering analysis demonstrated separation of normal from cancer cells although the separation was incomplete (Figure 4.3). The four colon cancer cell lines were grouped into two sub-clusters, sub-cluster 1 was represented by HT-29 and WiDr cells and sub-cluster 2 was represented by HCT-15 and SK-CO-1 cells. Interestingly, HT-29 and WiDr cells were found derived from low and non-metastatic colon cancers, whereas HCT-15 and SK-CO-1 cells were derived from high metastatic or high invasive potential colon cancer (Shen et al., 2000; Wang et al., 2003; de Toledo et al., 2012). These observations were possibly the reasons that had led to similar

miRNA expression profiles of HT-29 - WiDr cells and HCT-15 - SK-CO-1 cells, and thus, two sub-clusters observed.

Table 5.1: Summary of miRNA expression in colon cancer (Panarelli and Yantiss, 2011).

Study	Differentially expressed miRNAs (no./total)	Most significantly overexpressed miRNAs in colon cancer	Most significantly underexpressed miRNAs in colon cancer
Bandres et al.	13/156	miR-31, miR-96, miR-133b, miR-135b, miR-145, miR-183	
Wang et al.	14/723	miR-106b, miR-135b, miR-18a, miR-18b, miR-196b, miR-19a, miR-224, miR-335, miR-424, miR-20a*, miR-301b, miR-734a	miR-378, miR-378*
Schepeler et al.	60/315	miR-20a, miR-510, miR-92, miR-513	miR-145, miR-455, miR-484, miR-101
Slaby et al.	4/4	miR-31, miR-21	miR-145, miR-143
Xi et al.	4/10	miR-15b, miR-181b, miR-191, miR-200c	
Schetter et al.	37/389	miR-20a, miR-21, miR-106a, miR-181b, miR-203	
Sarver et al.	39/735	miR-135b, miR-96, miR-182, miR-182*, miR-183	miR-1, miR-133a, miR-30a-3p, miR-30a-5p, miR-20b, miR-363
Michael et al.	2/28		miR-143, miR-145
Guo et al.	45/262	miR-93, miR-92, miR-520h, miR-508, miR-505, miR-449, miR-429, miR-384, miR-373, miR-34c, miR-326, miR-25, miR-224, miR-210, miR-200a, miR-19b, miR-19a, miR-18a, miR-183, miR-182, miR-181b, miR-181a, miR-181c, miR-17-5p, miR-148a, miR-141, miR-130b, miR-128a, miR-106b, miR-106a, let-7d	miR-96, miR-485-5p, miR-422b, miR-342, miR-214, miR-199a, miR-195, miR-150, miR-145, miR-143, miR-133a, miR-126, miR-125b, miR-100

Numerous studies have reported that only one strand (either 5p or 3p arm) of the RNA duplex is selected to enter and be assembled with the RNA-induced silencing complex (RISC) and is, therefore, expressed in abundance as a regulatory element. On the other hand, the alternative strand – the passenger strand or miRNA* strand, is degraded upon unwinding of the miRNA duplex (Alexiou et al., 2010; Guo and Lu, 2010; Yang et al., 2011; Pahl et al., 2012). The identified alternative miRNA* species is often neglected or not included in further downstream analysis as it is generally degraded upon miRNA assembling to the RISC complex (Mah et al., 2010; Pahl et al., 2012). Co-expression of both the miRNA-5p and -3p species has recently been started to be recognised and evidences show the abundance, possible functions, and physiological relevance of miRNA* species in cancers or other diseases (Jazdzewski et al., 2009; Guo and Lu, 2010; Jiang et al., 2010; Lin et al., 2010; Mah et al., 2010; Jin et al., 2011; Almeida et al., 2012).

Mature selection of miRNA-5p or -3p strand to be incorporated into RISC is influenced by relative thermodynamic stability of the two ends of the miRNA duplex. In most cases, the functional miRNA strand with less-stable base pairing at the 5’ end typically evades degradation during mature strand selection (Mah et al., 2010). This finding is supported by the report that a less stable 5’ end preferentially incorporates itself into the RISC and hence, to efficient mRNA targeting and degradation (Schuck et al., 2004; Barrero et al., 2011).

The co-expression of miRNA-5p and -3p pair might be due to similar thermodynamic stability and energy at both termini of the miRNA duplex which further leads to little or no thermodynamic bias for the mature-strand selection of either of the miRNA duplex ends (Mah et al., 2010) and, therefore, both miRNA-5p and -3p are co-expressed. However, the exact mechanism co-expression of miRNA pairs requires further investigation.

In this study, a total of 19 pairs miRNA-5p and -3p (38 miRNAs in total) were revealed to be significantly co-expressed in similar expression pattern (Table 4.5), in which the regulation patterns of miRNA pairs are consistent with data of previous publications summarised in Table 5.1, e.g. in Sarver et al., miR-182 and miR-182* are reported co-up-regulated in colon cancer. Besides, miR-20a pair was significantly up-regulated in colon cancer cells in this study (Table 4.5), which is consistent with previous report (Table 5.1). On the other hands, miR-7 and let-7i pairs were significantly up-regulated in colon cancer cells (Table 4.5), which have never been reported in other colon cancer-related studies.

5.2 miRNAs Involved in Colon Cancer Pathogenesis-related Pathways

Dysregulation of miRNAs may contribute to the carcinogenesis process if the miRNA targets are genes coding for tumour suppressor protein or oncogenic protein (Shenouda and Alahari, 2009; Slaby et al., 2009).

miRNAs that are up-regulated or overexpressed in cancer may function as oncogenes, commonly referred to as oncogenic miRNA, or oncomiR (Shenouda and Alahari, 2009). The up-regulation of oncogenic miRNAs may result from transcriptional activation or amplification of miRNA encoding genes, promoting cancer-associated phenotypes and sustaining growth of cancer cells (Slaby et al., 2009). On the other hand, miRNAs with tumour suppressor activities in normal tissue are down-regulated or underexpressed in cancer, and are commonly referred to as tumour suppressive miRNAs (Shenouda and Alahari, 2009). Down-regulation of tumour suppressive miRNAs may result from chromosomal deletion, mutation, epigenetic silencing or miRNA processing alterations, thus initiating malignant transformation of normal cells (Slaby et al., 2009). Roles of oncogenic and tumour suppressive miRNAs in carcinogenesis process are shown in Figure 5.1 (Paranjape et al., 2009). Elevated levels of oncogenic miRNAs in cancer cells assemble and block translation of the targeted tumour suppressor genes, while reduced levels of tumour suppressive miRNAs are unable to assemble themselves onto the transcripts of their targeted oncogenes, therefore oncogenic proteins are translated (Paranjape et al., 2009). The joint effects of aberrant oncogenic and tumour suppressive miRNAs levels lead to initiation of the carcinogenesis process in cells (Paranjape et al., 2009).

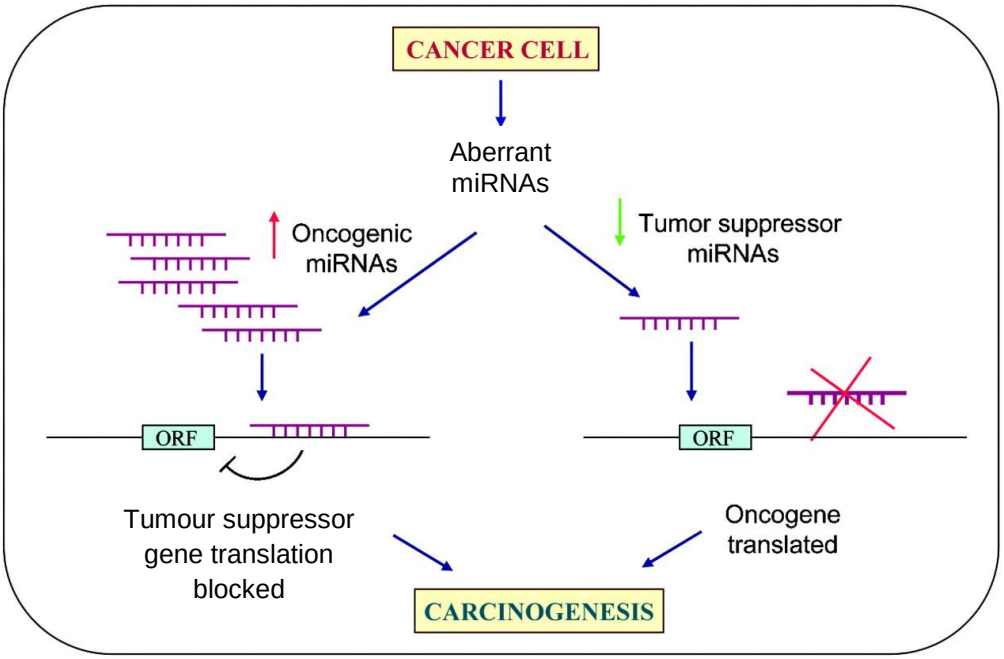


Figure 5.1: miRNA may have oncogenic or tumour suppressive properties in carcinogenesis (Paranjape et al., 2009).

Oncogenic miRNAs are frequently up-regulated in cancer to promote cancer-related phenotypes, i.e. 92 up-regulated miRNAs (71.9%) identified in colon cancer cells (Table 4.2). Tumour suppressive miRNAs are often found to be down-regulated in cancer, i.e. 36 down-regulated miRNAs (28.1%) identified in colon cancer cells. Amongst the differentially expressed miRNAs, 71.9% of them are oncogenic miRNAs, only 28.1% of them are tumour suppressive miRNAs. In this study, more oncogenic miRNAs are detected. Hence, we hypothesised that oncogenic miRNAs contribute more to carcinogenesis via down-regulating tumour suppressor genes encoding proteins, such as TP53 and PTEN to promote cancer; minority numbers of tumour suppressive miRNAs are contributed to carcinogenesis via up-regulating oncogenic proteins, such as JUN and MYC.

In Vogelstein's multistep colon carcinogenesis model (Figure 5.2), miRNAs are showed to be contributed to the regulation of various key colon cancer-related oncogenic and tumour suppressor pathways (Slaby et al., 2009). Numerous proteins involved in Wnt/β-catenin pathway, EGFR signalling and its downstream KRAS and phosphatidylinositol-3-kinase (PI3K) pathways, extracellular matrix (ECM) breakdown and epithelial-mesenchymal transition (EMT) pathways, p53 induction of cell cycle checkpoints and apoptosis, are altered and probably affected by the involvement of miRNA in colon carcinogenesis (Figure 5.2) (Slaby et al., 2009).

We attempt to integrate miRNAs identified in this study into the multistep colon carcinogenesis model. It is observed that a number of miRNAs that differentially expressed this study are involved in the early stages of carcinogenesis, including miR-145, miR-21, miR-17-92, miR-143, miR-18a and let-7. Hence, these miRNAs are served as potential candidates for diagnostic signature array development for early stage detection of colon cancer.

The miR-17-92 cluster, including miR-17, miR-18a, miR-20a pairs were up-regulated in this study. The expression levels of these miRNAs in colon cancer cells are consistent with previous study (Figure 5.2) (Slaby et al., 2009; Goel and Boland, 2012). This miRNA family is reported to be involved in regulating the major pathway β-catenin/WNT signalling that drive the progression of colon carcinogenesis (Slaby et al., 2009; Goel and Boland, 2012). Another miRNA associated with β-catenin/WNT signalling regulation is miR-145, which is targeted on MYC protein. miR-145 was revealed to be down-regulated in this study, which is also consistent with previous study (Figure 5.2) (Slaby et al., 2009; Goel and Boland, 2012).

In this study, miR-143 and let-7d-3p were down-regulated in colon cancer cells, echoing with previous study (Figure 5.2). Slaby et al. (2009) reported that they were involved in the regulation of EGFR signalling pathway, targeting on KRAS protein, blocking the constitutive phosphorylation of

MAPK. Besides, miR-21 was found significantly up-regulated in colon cancer cells in this study, which is reported strongly repressing tumour suppressor gene PTEN, associated with the augmentation of PI-3-K signalling and the progression of colon cancer (Slaby et al., 2009; Goel and Boland, 2012).

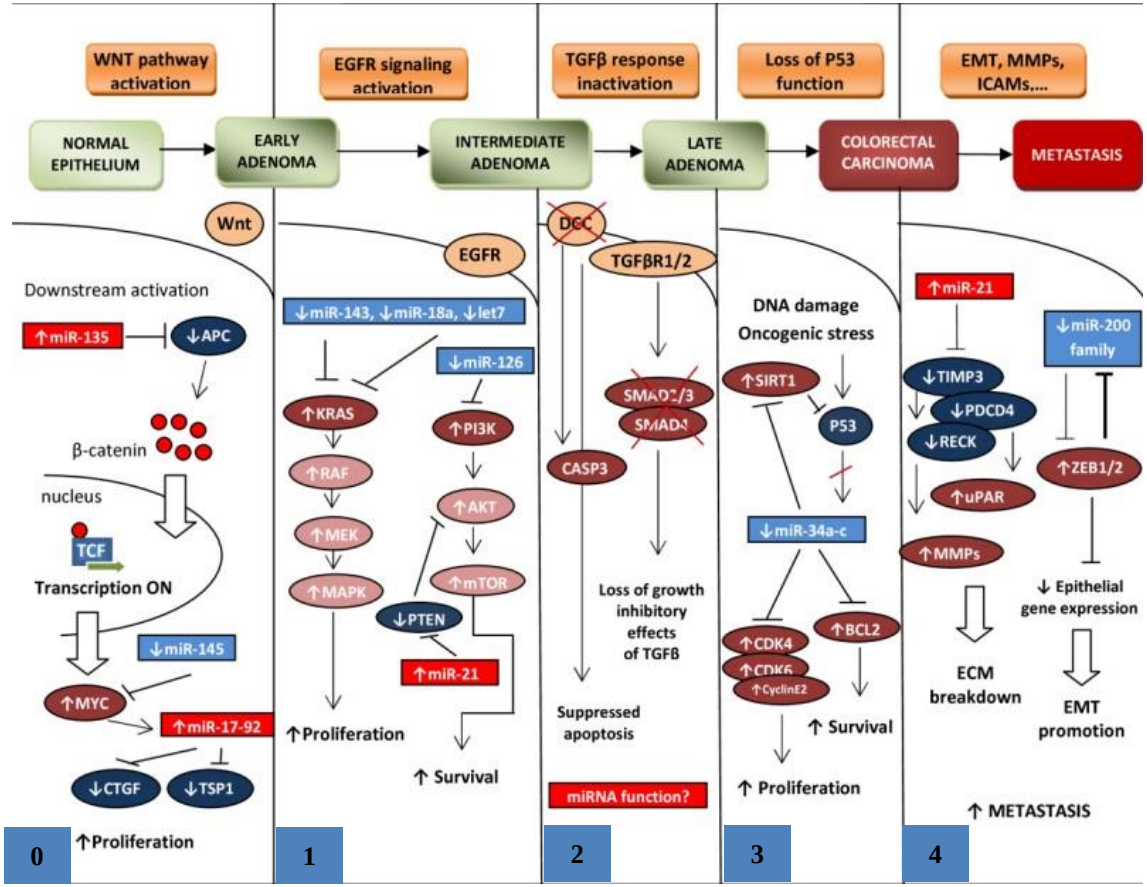


Figure 5.2: Involvement of miRNA in Vogelstein's multistep model of colon carcinogenesis targeted on various oncogenic and tumour suppressive pathways (Slaby et al., 2009).

Through investigating putative target pathways of the differentially miRNAs identified in this study, these miRNAs are found to be strongly related to colon cancer-related regulatory pathways, including apoptosis, cell cycle, mitogen-activated protein kinase (MAPK) signalling cascade, p53 signalling pathway and EMT (Table 4.3 and 4.7), complement the involvement of miRNAs in Vogelstein's multistep colon carcinogenesis model (Slaby et al., 2009) and potentially serve as the diagnostic biomarkers and possibly the therapeutic targets for colon cancer (Reid et al., 2012). In this study, a comprehensive theoretical analysis of the relationship between miRNAs and the selected target pathways was performed. This provided the basis for elucidation of the regulatory mechanism of miRNAs on its targeted pathways, taking a further step towards the understanding of colon carcinogenesis. By elucidating the colon carcinogenesis pathway, miRNA biomarkers for the development of an improved diagnostic tool is anticipated to be discovered, particularly targeting at stage 0 to stage I for identifying precursor lesions, polyps and benign tumour, so that proper treatment may be applied more timely leading to better prognosis (Figure 5.2).

5.3 Cross Targeting of let-7d-5p and -3p Pair on *IGF1R* and *KRAS* Involved in Apoptosis

Through bioinformatics analysis to predict miRNA target gene transcripts, it is noted that the validated targets (targets with hash ($^{\#}$) in Table 4.7) were clustered either for the miRNA-5p or -3p species, suggesting that

they were targeted by the major strand miRNA species. The predicted targets (targets without hash) were likely the lower-abundant alternative-strand miRNA species that await experimental validation.

In general, members from the same miRNA family are predicted to possess similar functions and regulate specific pathways as they are found to be targeted on similar set of gene transcripts due to identical seed sequences. Besides, multiple miRNAs, or miRNA-5p and 3p pair derived from the same precursor, with different sequences may be targeting at the same transcript, and this is possible through miRNA recognition of different binding sites of the same transcript. On the other hand, the same miRNA may cross-target different transcripts encoding for proteins of related or similar functions in the carcinogenesis processes to defend against alterations of miRNA regulatory pathways. In the case of one gene is being affected or knocked-down in the pathway, another gene targeted by the same or other miRNAs would still be available to regulate and maintain the miRNA regulatory circuit and to exert related phenotypic function.

Initial bioinformatics analysis predicted that *IGF1R* mRNA transcript was targeted by let-7d-5p and *KRAS* transcript was targeted by let-7d-3p. Validation of these predicted target gene was achieved by miRNA transfection assays by using specific miRNA inhibitor or mimic. Real-time PCR and western blot data supported regulation of IGF1R by let-7d-5p via increasing

mRNA level and protein synthesis (Figure 5.3) (Guo et al., 2010). Likewise, let-7d-3p regulates KRAS via decreasing *KRAS* mRNA level and increasing degradation of KRAS transcript (Figure 5.4) (Guo et al., 2010). The question whether the changes in let-7d target transcripts and proteins are achieved through a direct post-transcriptional regulation by let-7d miRNA or indirect regulation through other unknown genes requires further investigation.

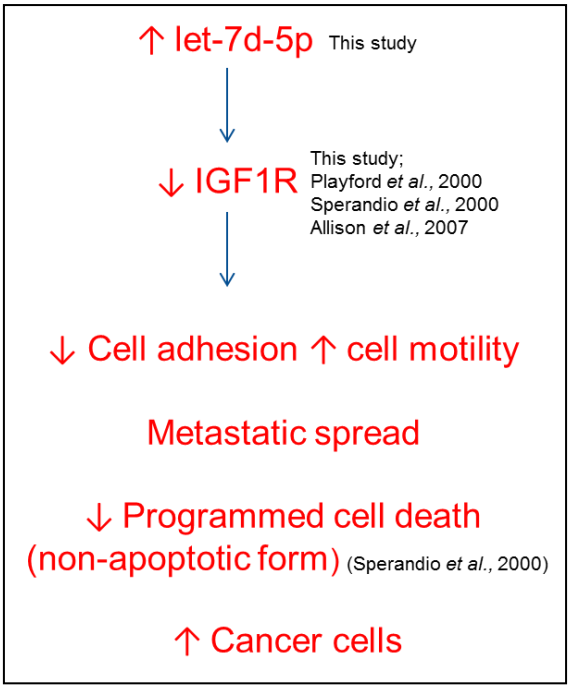


Figure 5.3: A proposed model of let-7d-5p – IGF1R contribution to colon carcinogenesis.

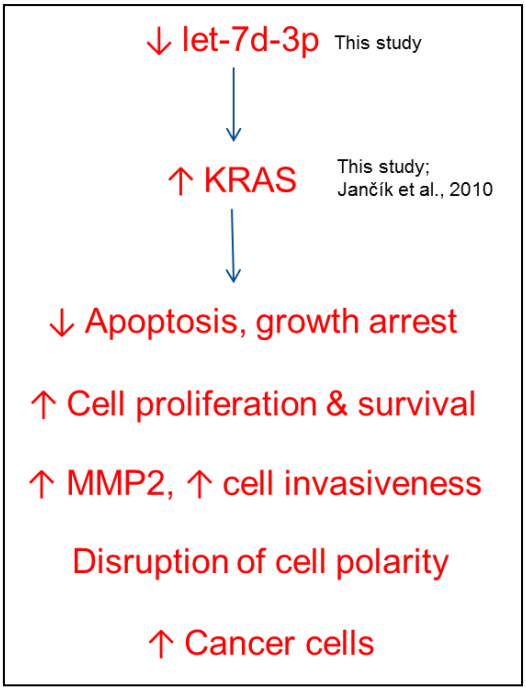


Figure 5.4: A proposed model of let-7d-3p – KRAS contribution to colon carcinogenesis.

In this study, let-7d-5p was found to be up-regulated in colon cancer cells and let-7d-5p was proposed to be inhibiting or down-regulating IGF1R in colon cancer cells. Correspondingly, our data showed that *IGF1R* mRNA and protein levels were decreased in colon cancer cells relative to normal colonic cells, consistent with previous report of down-regulation of IGF1R protein in colon cancer cell lines HT-29 and LoVo cells (Playford et al., 2000; Jones et al., 2006; Brierley et al., 2010). A previous report has also showed that the IGF1R protein is up-regulated in early-stage tumours but is down-regulated in advanced-stage colon cancer (Allison et al., 2007), breast cancer (Schnarr et al., 2000) and prostate cancer (Hellawell et al., 2002). IGF1R protein is widely recognised as promoting malignant transformation of normal cells. The colon cancer cells with decreased IGF1R protein levels were differentiated into mesenchymal-type cells and showed decreased cell adhesion and increased cell motility (Allison et al., 2007). These changes could favour the metastatic spread of cancer cells to distant lymph nodes or organs. Besides, decreased IGF1R levels are also associated with decreased levels of non-apoptotic form of programmed cell death (Sperandio et al., 2000), and hence, increased the invasiveness of cancer cells.

On the other hand, let-7d-3p was found to be down-regulated in colon cancer cells. It was proposed that decreased level of let-7d-3p targeted on *KRAS* mRNA and, thus, up-regulating KRAS protein in colon cancer cells. Correspondingly, our data showed that the KRAS protein was increased in colon cancer cells relative to cells derived from normal colon tissues,

consistent with a previous report (Jancik et al., 2010). Wild-type *KRAS* gene is originally a tumour suppressor gene in normal cells. However, *KRAS* is frequently mutated during tumour progression of cancer (Brink et al., 2003; Liu et al., 2011b). The mutated *KRAS* gene is often activated in colon cancer cells (Brink et al., 2003; Jancik et al., 2010). The activated *KRAS* gene possesses oncogenic properties and is expressed in elevated levels; it is also commonly involved in the development of various human cancers, including colon cancer (Hiyama et al., 2002; Jancik et al., 2010). Cells with elevated KRAS protein level are often associated with decreased apoptosis cell death and growth arrest, and at the same time increased cell proliferation and survival rate of cancer cells. Elevated expression of activated *KRAS* gene was revealed to enhance metalloproteinase 2 (MMP2) in the matrix to enhance invasiveness of cancer cells. Besides, elevated levels of KRAS protein disrupt the cell polarity, a hallmark of cancer (Wang et al., 2009), which are responsible for the diversification of cell shapes but also for regulation of asymmetric cell divisions of stem cells that are crucial for their correct self-renewal and differentiation (Jancik et al., 2010).

5.4 Potential Diagnostic, Prognostic and Therapeutics Applications of miRNA for Colon Cancer

miRNA expression profiles were shown to have correlation with tumour origin in previous reports, allowing characterisation of tumours of unknown origin and indistinguishable by histology (Farazi et al., 2013).

Advances in miRNA detection, such as real-time qRT–PCR, may allow miRNAs to be used as diagnostic and prognostic markers in the clinic by detecting circulating miRNA in blood plasma or stool of colon cancer patients (Zhai and Ju, 2011), and for discriminating colon cancer from gastric cancer and inflammatory bowel diseases (IBD) by using cancer-specific miRNA biomarker miR-92a (Schee et al., 2010).

Association between dysregulation of miRNA and the outcome of disease has been demonstrated. Elevated expression of miR-21 is associated with the development of distant metastases with advanced tumour, lymph node and metastasis staging (Yan et al., 2008). On the other hand, miRNAs are also specifically associated with increased tumour size and metastasis. For example, low abundance of miR-143 and miR-145 are associated with tumour size larger than 50 mm (Zarate et al., 2012). Besides, miRNA gene or miRNA binding site polymorphisms and miRNA gene expression alterations are reported to be associated with treatment outcome of colon cancer (Wu et al., 2011). For example, a polymorphism in let-7 miRNA binding site at *KRAS* 3'-UTR is showing positive correlation with cetuximab responsiveness in wild-type KRAS metastatic colon cancer patients (Zhang et al., 2011b). Similarly, the expression levels of let-7g and miR-181b in colon cancer are significantly associated with chemo-responses to S-1 (5-fluorouracil-based antimetabolite) (Nakajima et al., 2006).

Gene therapy using miRNAs might be an effective approach when combined with chemotherapy to inhibit tumour progression (Lujambio and Lowe, 2012). Manipulation of specific miRNA levels in cancer cells using miRNA replacement (mimics) or anti-miRNA (inhibitors) can restore miRNA activities and repair the damaged gene regulatory network and signalling pathways (Figure 5.5) to reverse the phenotypes of cancerous cells (Yu et al., 2013). New advances in delivery of miRNA inhibitors and mimics hold the promise of rapidly translating knowledge of miRNAs into disease treatment. In this study, we revealed that the expression levels of let-7d-5p and -3p affected the mRNA and protein expression levels of IGF1R and KRAS, in which both transcripts are associated with cell death, in apoptotic or non-apoptotic form (Figures 5.3 and 5.4). Theoretically, cell invasiveness and could be decreased and cancer cell death could be increased by increasing the IGF1R protein level and decreasing the KRAS protein level through manipulation of let-7d levels. However, more specific assays to confirm the phenotypic effects of let-7d targeting IGF1R and KRAS as well as detailed pharmacokinetics-pharmacodynamics studies are required to determine the optional dosage of miRNA oligonucleotides to be used safely (Garzon et al., 2009). Finally, the precursor generating miRNA-5p and -3p species may each has distinct clinical effects (Almeida et al., 2012). Therefore, manipulating miRNA expression for therapeutic purposes using precursor miRNA should be carried out with caution.

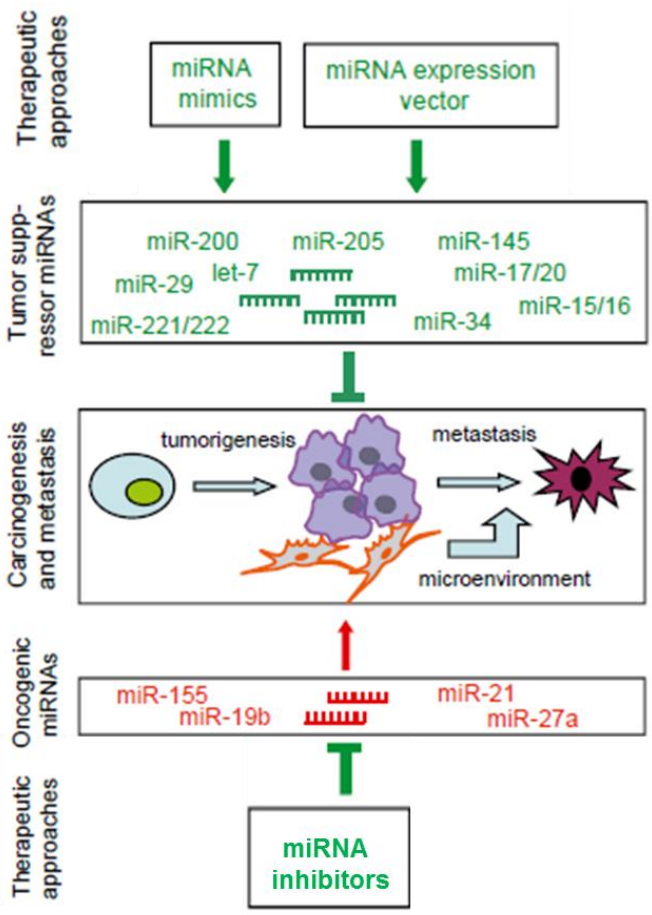


Figure 5.5: Clinical applications of miRNAs in cancer therapy (Yu et al., 2013).

5.5 Future Work

Analysis focusing on other miRNA-5p and 3p pairs and their targets (Table 4.7) will be performed in other studies to confirm the predicted targets derived from the miRNA target prediction algorithm and their putative functions. Other significantly expressed miRNAs, which do not occur in the 5p/3p pairs, in colon cancer cells, and their respective novel targets are also worthy of further investigation in future studies. More importantly, future

investigation will be carried out using normal-cancer paired clinical biopsies obtained from colon cancer patients to further elucidate possible functional contributions of co-expression miRNA-5p and -3p pairs in colon cancer pathogenesis. Clinically validated miRNAs that are differentially expressed in cancer relative to normal tissues are useful in the development of a diagnostic signature array chip for early detection of colon cancer in Malaysia.

CHAPTER 6

CONCLUSIONS

By using colon cancer as a study model, miRNA expression profile of colon cancer cells was established in this study. A total of 128 miRNAs are differentially expressed in colon cancer cells were successfully identified indicating significant miRNA dysregulation in colon cancer cells. More than 2/3 of dysregulated miRNAs identified in the study are found to be up-regulated, indicating miRNAs play a more important role in repressing or inhibiting tumour suppressor proteins rather than in up-regulating oncogenic proteins in the carcinogenesis process.

The miRNA profiling has also led to the finding of frequent miRNA-5p and -3p co-expression in colon cancer cells and most of the co-expressed miRNA pairs were co-up-regulated or co-down-regulated. Mature strand selection of either one or both the 5p and 3p strands could be associated with alteration of miRNA levels, external signals that triggered intracellular changes in protein-protein interaction and, thus, affecting the availability of target transcripts. On the other hand, through bioinformatics analysis, both the miRNA-5p and -3p species derived from the same miRNA precursor are mostly found to be targeting different transcripts and affecting the same or different biological pathways; the same miRNA may also cross-target different mRNA transcripts of the same biological pathway. Occasionally,

miRNA-5p and -3p derived from the same miRNA precursor are found to target the same mRNA transcript.

The inverse co-expression of let-7d-5p and -3p pair was validated by miRNA real-time PCR. Furthermore, this pair of miRNAs was revealed to be targeting different putative targets IGF1R and KRAS that regulate the same cancer-related regulatory pathway - apoptosis. The miRNA inhibitor and mimic transfection assay results supported regulation of IGF1R by let-7d-5p by increasing IGF1R protein synthesis; let-7d-3p regulation of KRAS was via increasing degradation of KRAS transcripts. These results implicate that the miRNA pair could be functionally relevant to colon carcinogenesis.

Hence, the hypothesis is proven that both miRNA-5p and -3p are co-dysregulated in colon cancer cells, and miRNA dysregulation of 5p and 3p miRNA species is associated with altered target transcript expression that may be involved in important pathways in colon carcinogenesis.

Our results using cultured colon cancer cells provide the basis for future investigation using clinical specimen to elucidate possible functional contributions of co-expression miRNA-5p and -3p pairs in colon cancer pathogenesis. It is anticipated that at least 10 clinical specimen-validated pairs out of the 19 dysregulated miRNA pairs will be included into a chip and

further developed into diagnostic signature array for early detection and prognosis of human colon cancer.

REFERENCES

Ackroyd, F.W. and Hedberg, S.E., 1985. Colonic polyps. *Annual review of medicine*, 36 (1), pp. 619-625.

Ahearn, I.M., Haigis, K., Bar-Sagi, D. and Philips, M.R., 2012. Regulating the regulator: post-translational modification of RAS. *Nat Rev Mol Cell Biol*, 13 (1), pp. 39-51.

Aizat, A.A.A., Nurfatimah, M.S.S., Aminudin, M.M. and Ankathil, R., 2013. XPC Lys939Gln polymorphism, smoking and risk of sporadic colorectal cancer among Malaysians. *World J Gastroenterol*, 19 (23), pp. 3623-8.

Akao, Y. et al., 2010. Role of anti-oncomirs miR-143 and -145 in human colorectal tumors. *Cancer Gene Ther*, 17 (6), pp. 398-408.

Akao, Y., Nakagawa, Y. and Naoe, T., 2006. let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull*, 29 (5), pp. 903-6.

Al-Naggar, R.A. and Bobryshev, Y.V., 2013. Knowledge of colorectal cancer screening among young Malaysians. *Asian Pac J Cancer Prev*, 14 (3), pp. 1969-74.

Alberici, P., 2007, *The Adenoma-Carcinoma Sequence in Colorectal Cancer: scratching the surface.* PhD Thesis, Erasmus University Rotterdam, The Netherlands.

Alexiou, P. et al., 2010. miRGen 2.0: a database of microRNA genomic information and regulation. *Nucleic Acids Res*, 38 (Database issue), pp. D137-41.

Allison, A.S., McIntyre, M.A., McArdle, C. and Habib, F.K., 2007. The insulin-like growth factor type 1 receptor and colorectal neoplasia: insights into invasion. *Hum Pathol*, 38 (11), pp. 1590-602.

Almeida, M.I. et al., 2012. Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. *Gastroenterology*, 142 (4), pp. 886-896 e9.

Ariffin, O.Z. and Saleha, I.T.N., 2011. *Malaysia Cancer Statistics Data and Figure 2007.* Putrajaya: National Cancer Registry, Ministry of Health, Malaysia.

Arndt, G.M. et al., 2009. Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer. *BMC Cancer*, 9, pp. 374.

Aslam, M.I., Taylor, K., Pringle, J.H. and Jameson, J.S., 2009. MicroRNAs are novel biomarkers of colorectal cancer. *Br J Surg*, 96 (7), pp. 702-10.

Barrero, R.A. et al., 2011. Evolutionary conserved microRNAs are ubiquitously expressed compared to tick-specific miRNAs in the cattle tick Rhipicephalus (Boophilus) microplus. *BMC Genomics*, 12 (1), pp. 328.

Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116 (2), pp. 281-97.

Bartel, D.P., 2009. MicroRNAs: target recognition and regulatory functions. *Cell*, 136 (2), pp. 215-33.

Borralho, P.M. et al., 2009. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. *FEBS J*, 276 (22), pp. 6689-700.

Bray, F., Ren, J.S., Masuyer, E. and Ferlay, J., 2013. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int J Cancer*, 132 (5), pp. 1133-45.

Brierley, G.V. et al., 2010. Silencing of the insulin receptor isoform A favors formation of type 1 insulin-like growth factor receptor (IGF-IR) homodimers and enhances ligand-induced IGF-IR activation and viability of human colon carcinoma cells. *Endocrinology*, 151 (4), pp. 1418-27.

Brink, M. et al., 2003. K-ras oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study. *Carcinogenesis*, 24 (4), pp. 703-10.

Burk, U. et al., 2008. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Rep*, 9 (6), pp. 582-9.

Cai, C. et al., 2012. MicroRNA-211 expression promotes colorectal cancer cell growth in vitro and in vivo by targeting tumor suppressor CHD5. *PLoS One*, 7 (1), pp. e29750.

Calcagno, S.R. et al., 2008. Oncogenic K-ras promotes early carcinogenesis in the mouse proximal colon. *Int J Cancer*, 122 (11), pp. 2462-70.

Calin, G.A. and Croce, C.M., 2006. MicroRNA signatures in human cancers. *Nat Rev Cancer*, 6 (11), pp. 857-66.

Calin, G.A. et al., 2002. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A*, 99 (24), pp. 15524-9.

Caruso, S. et al., 2012. MicroRNAs in colorectal cancer stem cells: new regulators of cancer stemness? *Oncogenesis*, 1 (11), pp. e32.

Cascione, L. et al., 2013. Integrated microRNA and mRNA signatures associated with survival in triple negative breast cancer. *PLoS One*, 8 (2), pp. e55910.

Castanotto, D., Lingeman, R., Riggs, A.D. and Rossi, J.J., 2009. CRM1 mediates nuclear-cytoplasmic shuttling of mature microRNAs. *Proc Natl Acad Sci U S A*, 106 (51), pp. 21655-9.

Chen, X. et al., 2009. Role of miR-143 targeting KRAS in colorectal tumorigenesis. *Oncogene*, 28 (10), pp. 1385-92.

Chen, Y.B. 2012. Colon cancer. *In:* Zieve, D., Eltz, D.R., Slon, S. and Wang., N. (eds.) *A.D.A.M. Medical Encyclopedia*.

Choo, K.B., 2011. Epigenetics in disease and cancer. *Malays J Pathol*, 33 (2), pp. 61-70.

Christensen, B.C. et al., 2009. A let-7 microRNA-binding site polymorphism in the KRAS 3' UTR is associated with reduced survival in oral cancers. *Carcinogenesis*, 30 (6), pp. 1003-7.

Cummings, L.C. and Cooper, G.S., 2011. Colorectal cancer screening: update for 2011. *Semin Oncol*, 38 (4), pp. 483-9.

de Toledo, M., Anguille, C., Roger, L., Roux, P. and Gadea, G., 2012. Cooperative anti-invasive effect of Cdc42/Rac1 activation and ROCK inhibition in SW620 colorectal cancer cells with elevated blebbing activity. *PLoS One*, 7 (11), pp. e48344.

Desch, C.E. et al., 1999. Recommended colorectal cancer surveillance guidelines by the American Society of Clinical Oncology. *Journal of clinical oncology*, 17 (4), pp. 1312-1312.

Dong, Y. et al., 2011. MicroRNA dysregulation in colorectal cancer: a clinical perspective. *Br J Cancer*, 104 (6), pp. 893-8.

Eichner, L.J. et al., 2010. miR-378(*) mediates metabolic shift in breast cancer cells via the PGC-1beta/ERRgamma transcriptional pathway. *Cell Metab*, 12 (4), pp. 352-61.

Esquela-Kerscher, A. and Slack, F.J., 2006. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer*, 6 (4), pp. 259-69.

Farazi, T.A., Hoell, J.I., Morozov, P. and Tuschl, T., 2013. MicroRNAs in human cancer. *Adv Exp Med Biol*, 774, pp. 1-20.

Ferlay, J. et al., 2010, *GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10* [Online]. Available at: http://globocan.iarc.fr [Accessed: 10 January 2013].

Fernandez, E. et al., 2004. Family history and environmental risk factors for colon cancer. *Cancer Epidemiol Biomarkers Prev*, 13 (4), pp. 658-61.

Fijten, G.H. et al., 1995. Predictive value of signs and symptoms for colorectal cancer in patients with rectal bleeding in general practice. *Fam Pract*, 12 (3), pp. 279-86.

Galasso, M., Sana, M.E. and Volinia, S., 2010. Non-coding RNAs: a key to future personalized molecular therapy? *Genome Med*, 2 (2), pp. 12.

Garzon, R., Calin, G.A. and Croce, C.M., 2009. MicroRNAs in Cancer. *Annu Rev Med*, 60, pp. 167-79.

Geiger, T.M. and Ricciardi, R., 2009. Screening options and recommendations for colorectal cancer. *Clin Colon Rectal Surg*, 22 (4), pp. 209-17.

Goedeke, L. et al., 2013. A regulatory role for microRNA 33* in controlling lipid metabolism gene expression. *Mol Cell Biol*, 33 (11), pp. 2339-52.

Goel, A. and Boland, C.R., 2012. Epigenetics of colorectal cancer. *Gastroenterology*, 143 (6), pp. 1442-1460 e1.

Grady, W.M. and Tewari, M., 2010. The next thing in prognostic molecular markers: microRNA signatures of cancer. *Gut*, 59 (6), pp. 706-8.

Gregersen, L.H. et al., 2010. MicroRNA-145 targets YES and STAT1 in colon cancer cells. *PLoS One*, 5 (1), pp. e8836.

Guo, H., Ingolia, N.T., Weissman, J.S. and Bartel, D.P., 2010. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, 466 (7308), pp. 835-40.

Guo, L. and Lu, Z., 2010. The fate of miRNA* strand through evolutionary analysis: implication for degradation as merely carrier strand or potential regulatory molecule? *PLoS One*, 5 (6), pp. e11387.

Ha, T.Y., 2011. MicroRNAs in Human Diseases: From Cancer to Cardiovascular Disease. *Immune Netw*, 11 (3), pp. 135-54.

Haggar, F.A. and Boushey, R.P., 2009. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clin Colon Rectal Surg*, 22 (4), pp. 191-7.

He, J. et al., 2010. miRNA-mediated functional changes through co-regulating function related genes. *PLoS One*, 5 (10), pp. e13558.

Hellawell, G.O. et al., 2002. Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. *Cancer Res*, 62 (10), pp. 2942-50.

Hiyama, T. et al., 2002. K-ras mutation in helicobacter pylori-associated chronic gastritis in patients with and without gastric cancer. *Int J Cancer*, 97 (5), pp. 562-6.

Hu, M. et al., 2010. MicroRNA-141 regulates Smad interacting protein 1 (SIP1) and inhibits migration and invasion of colorectal cancer cells. *Dig Dis Sci*, 55 (8), pp. 2365-72.

Huang, Z. et al., 2010. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *Int J Cancer*, 127 (1), pp. 118-26.

Hunter, M.P. et al., 2008. Detection of microRNA expression in human peripheral blood microvesicles. *PLoS One*, 3 (11), pp. e3694.

Iliopoulos, D., Jaeger, S.A., Hirsch, H.A., Bulyk, M.L. and Struhl, K., 2010. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Mol Cell*, 39 (4), pp. 493-506.

Jancik, S., Drabek, J., Radzioch, D. and Hajduch, M., 2010. Clinical relevance of KRAS in human cancers. *J Biomed Biotechnol*, 2010, pp. 150960.

Jazdzewski, K. et al., 2009. Polymorphic mature microRNAs from passenger strand of pre-miR-146a contribute to thyroid cancer. *Proc Natl Acad Sci U S A*, 106 (5), pp. 1502-5.

Jiang, L. et al., 2010. Hsa-miR-125a-3p and hsa-miR-125a-5p are downregulated in non-small cell lung cancer and have inverse effects on invasion and migration of lung cancer cells. *BMC Cancer*, 10 (1), pp. 318.

Jin, L. et al., 2011. MicroRNA-149*, a p53-responsive microRNA, functions as an oncogenic regulator in human melanoma. *Proc Natl Acad Sci U S A*, 108 (38), pp. 15840-5.

Jones, H.E. et al., 2006. Inhibition of insulin receptor isoform-A signalling restores sensitivity to gefitinib in previously de novo resistant colon cancer cells. *Br J Cancer*, 95 (2), pp. 172-80.

Kanehisa, M. and Goto, S., 2000. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res*, 28 (1), pp. 27-30.

Kanehisa, M. et al., 2014. Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res*, 42 (Database issue), pp. D199-205.

Kim, M.S., Lee, J. and Sidransky, D., 2010. DNA methylation markers in colorectal cancer. *Cancer Metastasis Rev*, 29 (1), pp. 181-206.

Kosaka, N., Iguchi, H. and Ochiya, T., 2010. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*, 101 (10), pp. 2087-92.

Kozomara, A. and Griffiths-Jones, S., 2014. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*, 42 (Database issue), pp. D68-73.

Kuchenbauer, F. et al., 2011. Comprehensive analysis of mammalian miRNA* species and their role in myeloid cells. *Blood*, 118 (12), pp. 3350-3358.

Kusenda, B., Mraz, M., Mayer, J. and Pospisilova, S., 2006. MicroRNA biogenesis, functionality and cancer relevance. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*, 150 (2), pp. 205-15.

Lai, X. et al., 2012. Computational analysis of target hub gene repression regulated by multiple and cooperative miRNAs. *Nucleic Acids Res*, 40 (18), pp. 8818-34.

Landi, D. et al., 2008. Polymorphisms within micro-RNA-binding sites and risk of sporadic colorectal cancer. *Carcinogenesis*, 29 (3), pp. 579-84.

Lee, R.C., Feinbaum, R.L. and Ambros, V., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75 (5), pp. 843-54.

Lee, Y., Jeon, K., Lee, J.T., Kim, S. and Kim, V.N., 2002. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*, 21 (17), pp. 4663-70.

Leng, Z. et al., 2013. Kruppel-like factor 4 acts as an oncogene in colon cancer stem cell-enriched spheroid cells. *PLoS One*, 8 (2), pp. e56082.

Li, C., Feng, Y., Coukos, G. and Zhang, L., 2009. Therapeutic microRNA strategies in human cancer. *AAPS J*, 11 (4), pp. 747-57.

Lim, K.J., Bisht, S., Bar, E.E., Maitra, A. and Eberhart, C.G., 2011. A polymeric nanoparticle formulation of curcumin inhibits growth, clonogenicity and stem-like fraction in malignant brain tumors. *Cancer Biology & Therapy*, 11 (5), pp. 464-473.

Lim, L.P. et al., 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, 433 (7027), pp. 769-73.

Lin, R.J., Lin, Y.C. and Yu, A.L., 2010. miR-149* induces apoptosis by inhibiting Akt1 and E2F1 in human cancer cells. *Mol Carcinog*, 49 (8), pp. 719-27.

Liu, C. et al., 2011a. MiR-21 plays an important role in radiation induced carcinogenesis in BALB/c mice by directly targeting the tumor suppressor gene Big-h3. *Int J Biol Sci*, 7 (3), pp. 347-63.

Liu, L., Chen, L., Xu, Y., Li, R. and Du, X., 2010. microRNA-195 promotes apoptosis and suppresses tumorigenicity of human colorectal cancer cells. *Biochem Biophys Res Commun*, 400 (2), pp. 236-40.

Liu, X., Jakubowski, M. and Hunt, J.L., 2011b. KRAS gene mutation in colorectal cancer is correlated with increased proliferation and spontaneous apoptosis. *Am J Clin Pathol*, 135 (2), pp. 245-52.

López, J.A. and Alvarez-Salas, L.M., 2011. Differential effects of miR-34c-3p and miR-34c-5p on SiHa cells proliferation apoptosis, migration and invasion. *Biochemical and Biophysical Research Communications*, 409 (3), pp. 513-519.

Lu, J. et al., 2005. MicroRNA expression profiles classify human cancers. *Nature*, 435 (7043), pp. 834-8.

Lu, M. et al., 2008. An analysis of human microRNA and disease associations. *PLoS One*, 3 (10), pp. e3420.

Lujambio, A. and Lowe, S.W., 2012. The microcosmos of cancer. *Nature*, 482 (7385), pp. 347-55.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J.E. and Kutay, U., 2004. Nuclear export of microRNA precursors. *Science*, 303 (5654), pp. 95-8.

Lundstrom, K., 2011. Micro-RNA in disease and gene therapy. *Curr Drug Discov Technol*, 8 (2), pp. 76-86.

Luo, X., Burwinkel, B., Tao, S. and Brenner, H., 2011. MicroRNA signatures: novel biomarker for colorectal cancer? *Cancer Epidemiol Biomarkers Prev*, 20 (7), pp. 1272-86.

Ma, Y.L. et al., 2011. Human embryonic stem cells and metastatic colorectal cancer cells shared the common endogenous human microRNA-26b. *J Cell Mol Med*, 15 (9), pp. 1941-54.

MacFarlane, L.A. and Murphy, P.R., 2010. MicroRNA: Biogenesis, Function and Role in Cancer. *Curr Genomics*, 11 (7), pp. 537-61.

Magaji, B.A. et al., 2012. Health-related quality of life among colorectal cancer patients in Malaysia: a study protocol. *BMC Cancer*, 12 (1), pp. 384.

Mah, S.M., Buske, C., Humphries, R.K. and Kuchenbauer, F., 2010. miRNA*: a passenger stranded in RNA-induced silencing complex? *Crit Rev Eukaryot Gene Expr*, 20 (2), pp. 141-8.

Manavella, P.A., Koenig, D. and Weigel, D., 2012. Plant secondary siRNA production determined by microRNA-duplex structure. *Proc Natl Acad Sci U S A*, 109 (7), pp. 2461-6.

Michael, M.Z., SM, O.C., van Holst Pellekaan, N.G., Young, G.P. and James, R.J., 2003. Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res*, 1 (12), pp. 882-91.

Minardi, A.J., Jr., Sittig, K.M., Zibari, G.B. and McDonald, J.C., 1998. Colorectal cancer in the young patient. *Am Surg*, 64 (9), pp. 849-53.

Nakajima, G. et al., 2006. Non-coding MicroRNAs hsa-let-7g and hsa-miR-181b are Associated with Chemoresponse to S-1 in Colon Cancer. *Cancer Genomics Proteomics*, 3 (5), pp. 317-324.

Neugut, A.I. et al., 1993. Diagnostic yield of colorectal neoplasia with colonoscopy for abdominal pain, change in bowel habits, and rectal bleeding. *Am J Gastroenterol*, 88 (8), pp. 1179-83.

Ng, E.K. et al., 2009. MicroRNA-143 targets DNA methyltransferases 3A in colorectal cancer. *Br J Cancer*, 101 (4), pp. 699-706.

Nguyen, D.X., Bos, P.D. and Massague, J., 2009. Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer*, 9 (4), pp. 274-84.

O'Brien, C.A., Pollett, A., Gallinger, S. and Dick, J.E., 2007. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*, 445 (7123), pp. 106-10.

Ouyang, D.L., Chen, J.J., Getzenberg, R.H. and Schoen, R.E., 2005. Noninvasive testing for colorectal cancer: a review. *Am J Gastroenterol*, 100 (6), pp. 1393-403.

Ovcharenko, D., 2007, *MicroRNA and Cancer Therapeutics* [Online]. Available at: http://www.microrna.ws [Accessed: 19 January 2013].

Pahl, M.C. et al., 2012. MicroRNA expression signature in human abdominal aortic aneurysms. *BMC Med Genomics*, 5 (1), pp. 25.

Panarelli, N.C. and Yantiss, R.K., 2011. MicroRNA expression in selected carcinomas of the gastrointestinal tract. *Pathology research international*, 2011, pp. 124608.

Paranjape, T., Slack, F.J. and Weidhaas, J.B., 2009. MicroRNAs: tools for cancer diagnostics. *Gut*, 58 (11), pp. 1546-54.

Parasramka, M.A. et al., 2012. A role for low-abundance miRNAs in colon cancer: the miR-206/Kruppel-like factor 4 (KLF4) axis. *Clin Epigenetics*, 4 (1), pp. 16.

Pekow, J.R. et al., 2012. miR-143 and miR-145 are downregulated in ulcerative colitis: putative regulators of inflammation and protooncogenes. *Inflamm Bowel Dis*, 18 (1), pp. 94-100.

Playford, M.P., Bicknell, D., Bodmer, W.F. and Macaulay, V.M., 2000. Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. *Proc Natl Acad Sci U S A*, 97 (22), pp. 12103-8.

Puglisi, M.A., Tesori, V., Lattanzi, W., Gasbarrini, G.B. and Gasbarrini, A., 2013. Colon cancer stem cells: controversies and perspectives. *World J Gastroenterol*, 19 (20), pp. 2997-3006.

Rebollo, A., Perez-Sala, D. and Martinez, A.C., 1999. Bcl-2 differentially targets K-, N-, and H-Ras to mitochondria in IL-2 supplemented or deprived cells: implications in prevention of apoptosis. *Oncogene*, 18 (35), pp. 4930-9.

Reid, J.F. et al., 2012. miRNA profiling in colorectal cancer highlights miR-1 involvement in MET-dependent proliferation. *Mol Cancer Res*, 10 (4), pp. 504-15.

Ricci-Vitiani, L. et al., 2007. Identification and expansion of human colon-cancer-initiating cells. *Nature*, 445 (7123), pp. 111-5.

Romero-Cordoba, S. et al., 2012. Identification and pathway analysis of microRNAs with no previous involvement in breast cancer. *PLoS One*, 7 (3), pp. e31904.

Rossi, L., Bonmassar, E. and Faraoni, I., 2007. Modification of miR gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil in vitro. *Pharmacol Res*, 56 (3), pp. 248-53.

Ruby, J.G. et al., 2007. Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. *Genome Res*, 17 (12), pp. 1850-64.

Ryan, B.M., Robles, A.I. and Harris, C.C., 2010. Genetic variation in microRNA networks: the implications for cancer research. *Nat Rev Cancer*, 10 (6), pp. 389-402.

Sarver, A. et al., 2009. Human colon cancer profiles show differential microRNA expression depending on mismatch repair status and are characteristic of undifferentiated proliferative states. *BMC cancer*, 9 (1), pp. 401.

Sayed, D. and Abdellatif, M., 2011. MicroRNAs in development and disease. *Physiol Rev*, 91 (3), pp. 827-87.

Schee, K., Fodstad, O. and Flatmark, K., 2010. MicroRNAs as biomarkers in colorectal cancer. *Am J Pathol*, 177 (4), pp. 1592-9.

Schimanski, C.C. et al., 2009. High miR-196a levels promote the oncogenic phenotype of colorectal cancer cells. *World J Gastroenterol*, 15 (17), pp. 2089-96.

Schnarr, B. et al., 2000. Down-regulation of insulin-like growth factor-I receptor and insulin receptor substrate-1 expression in advanced human breast cancer. *Int J Cancer*, 89 (6), pp. 506-13.

Schopman, N.C., Heynen, S., Haasnoot, J. and Berkhout, B., 2010. A miRNA-tRNA mix-up: tRNA origin of proposed miRNA. *RNA Biol*, 7 (5), pp. 573-6.

Schuck, S., Manninen, A., Honsho, M., Fullekrug, J. and Simons, K., 2004. Generation of single and double knockdowns in polarized epithelial cells by retrovirus-mediated RNA interference. *Proc Natl Acad Sci U S A*, 101 (14), pp. 4912-7.

Seidel, S. et al., 2010. A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2 alpha. *Brain*, 133 (4), pp. 983-95.

Shen, J. et al., 2011. Plasma microRNAs as potential biomarkers for non-small-cell lung cancer. *Lab Invest*, 91 (4), pp. 579-87.

Shen, Y., Vogel, I. and Kalthoff, H., 2000. [Comparative study of metastasis-associated characteristics of tumor cells with different metastatic capacities]. *Zhonghua Zhong Liu Za Zhi*, 22 (3), pp. 201-4.

Shenouda, S.K. and Alahari, S.K., 2009. MicroRNA function in cancer: oncogene or a tumor suppressor? *Cancer Metastasis Rev*, 28 (3-4), pp. 369-78.

Siomi, H. and Siomi, M.C., 2009. On the road to reading the RNA-interference code. *Nature*, 457 (7228), pp. 396-404.

Slaby, O. et al., 2007. Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology*, 72 (5-6), pp. 397-402.

Slaby, O., Svoboda, M., Michalek, J. and Vyzula, R., 2009. MicroRNAs in colorectal cancer: translation of molecular biology into clinical application. *Molecular cancer*, 8 (1), pp. 102.

Sperandio, S., de Belle, I. and Bredesen, D.E., 2000. An alternative, nonapoptotic form of programmed cell death. *Proc Natl Acad Sci U S A*, 97 (26), pp. 14376-81.

Takaoka, Y. et al., 2012. Forced expression of miR-143 represses ERK5/c-Myc and p68/p72 signaling in concert with miR-145 in gut tumors of Apc(Min) mice. *PLoS One*, 7 (8), pp. e42137.

Tsang, W.P. and Kwok, T.T., 2009. The miR-18a* microRNA functions as a potential tumor suppressor by targeting on K-Ras. *Carcinogenesis*, 30 (6), pp. 953-9.

Tsutsumi, A., Kawamata, T., Izumi, N., Seitz, H. and Tomari, Y., 2011. Recognition of the pre-miRNA structure by Drosophila Dicer-1. *Nat Struct Mol Biol*, 18 (10), pp. 1153-8.

Valeri, N. et al., 2010. Modulation of mismatch repair and genomic stability by miR-155. *Proc Natl Acad Sci U S A*, 107 (15), pp. 6982-7.

Volinia, S. et al., 2010. Reprogramming of miRNA networks in cancer and leukemia. *Genome Res*, 20 (5), pp. 589-99.

Wang, M., Vogel, I. and Kalthoff, H., 2003. Correlation between metastatic potential and variants from colorectal tumor cell line HT-29. *World J Gastroenterol*, 9 (11), pp. 2627-31.

Wang, X. et al., 2011. Dynamic mechanisms for pre-miRNA binding and export by Exportin-5. *RNA*, 17 (8), pp. 1511-28.

Wang, X.Q. et al., 2009. Oncogenic K-Ras regulates proliferation and cell junctions in lung epithelial cells through induction of cyclooxygenase-2 and activation of metalloproteinase-9. *Mol Biol Cell*, 20 (3), pp. 791-800.

Weiland, M., Gao, X.H., Zhou, L. and Mi, Q.S., 2012. Small RNAs have a large impact: circulating microRNAs as biomarkers for human diseases. *RNA Biol*, 9 (6), pp. 850-9.

Wellner, U. et al., 2009. The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol*, 11 (12), pp. 1487-95.

Winter, J., Jung, S., Keller, S., Gregory, R.I. and Diederichs, S., 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol*, 11 (3), pp. 228-34.

Wu, W.K. et al., 2011. MicroRNA in colorectal cancer: from benchtop to bedside. *Carcinogenesis*, 32 (3), pp. 247-53.

Wu, Y. et al., 2013. Therapeutic delivery of MicroRNA-29b by cationic lipoplexes for lung cancer. *Molecular Therapy—Nucleic Acids*, 2 (4), pp. e84.

Yamakuchi, M. et al., 2010. P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. *Proc Natl Acad Sci U S A*, 107 (14), pp. 6334-9.

Yamamichi, N. et al., 2009. Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. *Clin Cancer Res*, 15 (12), pp. 4009-16.

Yan, L.X. et al., 2008. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA*, 14 (11), pp. 2348-60.

Yan, Z. et al., 2012. Integrative analysis of gene and miRNA expression profiles with transcription factor-miRNA feed-forward loops identifies regulators in human cancers. *Nucleic Acids Res*, 40 (17), pp. e135.

Yang, J.S. et al., 2011. Widespread regulatory activity of vertebrate microRNA* species. *RNA*, 17 (2), pp. 312-26.

Ye, J. et al., 2012. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics*, 13 (1), pp. 134.

Yi, R., Qin, Y., Macara, I.G. and Cullen, B.R., 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*, 17 (24), pp. 3011-6.

Yu, Z., Tozeren, A. and Pestell, R.G., 2013. Function of miRNAs in Tumor Cell Proliferation. In: Alahari, S. (ed.) *MicroRNA in Cancer*. The Netherlands: Springer, pp. 13-27.

Yusoff, H.M., Daud, N., Noor, N.M. and Rahim, A.A., 2012. Participation and barriers to colorectal cancer screening in Malaysia. *Asian Pac J Cancer Prev*, 13 (8), pp. 3983-7.

Zarate, R., Boni, V., Bandres, E. and Garcia-Foncillas, J., 2012. MiRNAs and LincRNAs: Could They Be Considered as Biomarkers in Colorectal Cancer? *Int J Mol Sci*, 13 (1), pp. 840-65.

Zeng, Y. and Cullen, B.R., 2003. Sequence requirements for micro RNA processing and function in human cells. *RNA*, 9 (1), pp. 112-23.

Zhai, H. and Ju, J., 2011. Implications of microRNAs in colorectal cancer development, diagnosis, prognosis, and therapeutics. *Frontiers in genetics*, 2 (78), pp. 00078.

Zhang, B., Pan, X., Cobb, G.P. and Anderson, T.A., 2007. microRNAs as oncogenes and tumor suppressors. *Dev Biol*, 302 (1), pp. 1-12.

Zhang, H. and Chen, Y., 2009. New insight into the role of miRNAs in leukemia. *Sci China C Life Sci*, 52 (3), pp. 224-31.

Zhang, Q. et al., 2011a. Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. *Cell Research*, 21 (4), pp. 579-587.

Zhang, W. et al., 2011b. A let-7 microRNA-binding site polymorphism in 3'-untranslated region of KRAS gene predicts response in wild-type KRAS patients with metastatic colorectal cancer treated with cetuximab monotherapy. *Ann Oncol*, 22 (1), pp. 104-9.

Zhang, Y. et al., 2012. MicroRNA-143 targets MACC1 to inhibit cell invasion and migration in colorectal cancer. *Molecular cancer*, 11 (1), pp. 23.

Zhu, L. et al., 2012. A functional polymorphism in miRNA-196a2 is associated with colorectal cancer risk in a Chinese population. *DNA Cell Biol*, 31 (3), pp. 350-4.

Appendix A

Supplementary Table 1: miRNAs that are activated or shutdown in colon cancer cells relative to normal colon cells

miRNA family	miRNA	Other name	$\log_2$(fold change)
		(a) Activated in colon cancer cells	
let-7	miR-98-5p	miR-98	4.45 ± 1.33**
mir-7	miR-7-5p	miR-7	6.44 ± 1.14**
mir-8	miR-200a-5p	miR-200a*	3.74 ± 2.77
mir-8	miR-200c-5p	miR-200c*	3.93 ± 1.05**
mir-8	miR-429	miR-429	5.27 ± 2.39*
mir-17	miR-20a-5p	miR-20a	7.19 ± 0.78**
mir-17	miR-20a-3p	miR-20a*	1.58 ± 0.98
mir-19	miR-19a-3p	miR-19a	3.61 ± 0.64**
mir-21	miR-21-3p	miR-21*	5.99 ± 0.85**
mir-29	miR-29b-1-5p	miR-29b-1*	2.93 ± 0.84**
mir-32	miR-32-5p	miR-32	4.80 ± 1.17**
mir-130	miR-301a-3p	miR-301a	3.32 ± 0.58**
mir-224	miR-224-5p	miR-224	6.74 ± 3.90
mir-335	miR-335-5p	miR-335	2.67 ± 1.39
mir-454	miR-454-3p	miR-454	3.56 ± 0.78**
mir-1204	miR-1204	miR-1204	2.66 ± 0.93**
-	miR-1274a	miR-1274a	5.77 ± 1.01**
-	miR-3679-3p	miR-3679-3p	3.39 ± 0.79**
-	miR-4304	miR-4304	6.14 ± 0.55**
		(b) Shutdown in colon cancer cells	
mir-15	miR-195-5p	miR-195	-2.11 ± 0.0008**
mir-143	miR-143-3p	miR-143	-8.00 ± 2.74**
mir-145	miR-145-5p	miR-145	-5.24 ± 2.16**
mir-199	miR-199a-5p	miR-199a	-3.84 ± 2.16**
mir-223	miR-223-3p	miR-223	-2.83 ± 0.001**
mir-342	miR-342-3p	miR-342	-6.87 ± 0.24**

Supplementary Table 1 continued:

miRNA family	miRNA	Alternative name	log_2(fold change)
		(b) Shutdown in colon cancer cells	
mir-370	miR-370-3p	miR-370	-6.83 ± 0.003**
mir-378	miR-378a-5p	miR-378*	-2.60 ± 0.003**
mir-451	miR-451a	miR-451	-2.70 ± 2.06*
mir-1268	miR-1268a	miR-1268	-3.51 ± 0.26**
-	miR-3195	miR-3195	-8.66 ± 0.16**
-	miR-4252	miR-4252	-4.84 ± 0.33**

Appendix B

Supplementary Table 2: Putative target transcripts and functions of the 19 co-expressed miRNA pairs in colon cancer cells

miRNA	Other name	Affected functions/processes: Selected target mRNAs
miRNA-5p/3p co-expression up-regulated		
let-7g-5p	let-7g	Angiogenesis: *IGF1R*[#], *MYC*[#], *TNFSF10* Apoptosis: *BCL2L1*, *IGF1R*[#], *MDM4*, *MYC*[#] Cell cycle: *MDM4* Inflammation: *TNFSF10* MAPK signalling pathway: *MYC*[#] p53 signalling pathway: *MDM4*
let-7g-3p	let-7g*	Angiogenesis: *ITGA1* Apoptosis: *EI24*, *ZAK* EMT: *ZEB1* MAPK signalling pathway: *NLK* Pyrimidine metabolism/ metabolic pathways: *CAD* Transcriptional misregulation in cancer: *ZEB1* Wnt signalling pathway: *NLK*
let-7i-5p	let-7i	Cell-cell signalling: *IL13*[#] Cytokine-cytokine receptor interaction: *IL13*[#] Inflammatory response: *IL13*[#], *TLR4*[#] Jak-STAT signalling pathway: *IL13*[#] Toll-like receptor signalling pathway: *TLR4*[#]
let-7i-3p	let-7i*	Apoptosis: *KPNA1* Cell cycle: *FOXG1*, *STAG2* Cell proliferation: *DLX5* Focal adhesion: *ACTB* Leukocyte transendothelial migration: *ACTB* Protein digestion and absorption: *COL12A1* Regulation of cytokine biosynthetic process: *IGFBP3* p53 signalling pathway: *IGFBP3*
miR-7-5p	miR-7	Angiogenesis: *CCND1*, *IGF1R*[#], *MYC*, *TIMP2* Apoptosis: *EGFR*[#], *IGF1R*[#], *MYC* Cell cycle: *CCND1* Cell growth: *SLC3A2* Cell migration: *EGFR*[#], *IRS1* DNA repair: *EGFR*[#] Inflammation: *SLC7A5* MAPK signalling pathway: *EGFR*[#] p53 signalling pathway: *CCND1* Response to drug: *ABCC1* Wnt signalling pathway: *CCND1*
miR-7-1-3p	miR-7-1*	Angiogenesis: *IGF1R* Apoptosis: *IGF1R* Inflammation: *JUN* MAPK signalling pathway: *JUN* Wnt signalling pathway: *JUN*

Supplementary Table 2 continued:

miRNA	Other name	Affected functions/processes: Selected target mRNAs
miRNA-5p/3p co-expression up-regulated		
miR-141-5p	miR-141*	Apoptosis: *ATM, NME1, ZAK* Cell adhesion molecules (CAMs): *CLDN1* Cell cycle: *ATM* Leukocyte transendothelial migration: *CLDN1* p53 signalling pathway: *ATM*
miR-141-3p	miR-141	Angiogenesis: *CCND1, ETS1, MAPK14, MET, MYC, PIK3CA, TP53* Apoptosis: *MAP3K10, MYC, PPIA, TP53, PTEN* Cell cycle: *CCND1, TP53* Drug target: *CDC25C, PPIA* EMT: *ZEB1*[#], *ZEB2*[#] Focal adhesion: *MET* Inflammation: *MAPK14, PPIA, TP53* MAPK signalling pathway: *MAPK14, MAP3K10, TP53* p53 signalling pathway: *CCND1, TP53, PTEN* Transcriptional misregulation in cancer: *ZEB1*[#] Wnt signalling pathway: *CCND1, TP53, ZEB2*[#]
miR-200a-5p	miR-200a*	Apoptosis: *BNIP1, FGF4, TIAM2* Cancer progression, invasion, metastasis: *TIAM2* Chromatin modification: *PRDM6* EMT: *ZEB2* MAPK signalling pathway: *FGF4* Wnt signalling pathway: *FZD1, PPAP2B, ZEB2*
miR-200a-3p	miR-200a	Angiogenesis: *ITGB3* Cell migration: *IRS2* Cell adhesion molecules (CAMs): *SELE* EMT: *ZEB1*[#], *ZEB2*[#] Inflammation: *SELE* Transcriptional misregulation in cancer: *ZEB1*[#] VEGF signalling pathway: *ITGB3* Wnt signalling pathway: *ZEB2*[#]
miR-17-5p	miR-17	Angiogenesis: *CCND1, THBS1*[#] Apoptosis: *E2F1*[#], *TGFBR2*[#], *PTEN*[#] Cell cycle: *CCND1, E2F1*[#], *RBL2, THBS1*[#] MAPK signalling pathway: *MAP3K2* Oxidative stress: *MAP3K2* p53 signalling pathway: *CCND1, THBS1*[#], *PTEN*[#] VEGF-R signalling pathway: *BMPR2* Wnt signalling pathway: *CCND1* EMT: *TGFBR2*[#], *SMAD4*
miR-17-3p	miR-17*	Angiogenesis: *CD44* Cancer: *CD44, MYB* Inflammation: *CD44, IL6ST* Proteolysis: *IFT52*
miR-18a-5p	miR-18a	Angiogenesis: *HIF1A* Apoptosis: *ATM, E2F1*[#], *TGFBR2*[#], *PTEN*[#] Cell cycle: *ATM, CCND2, CDK2, SMAD2, THBS1*[#] Focal adhesion: *CCND2,CDC42, MYLK, THBS1*[#] MAPK signalling pathway: *CDC42, TNFSF11* p53 signalling pathway: *ATM, CCND2, CDK2, THBS1*[#], *PTEN*[#] VEGF signalling pathway: *CDC42, HIF1A* Wnt signalling pathway: *CCND2, SMAD2* EMT: *TGFBR2*[#], *SMAD4*

Supplementary Table 2 continued:

miRNA	Other name	Affected functions/processes: Selected target mRNAs
miRNA-5p/3p co-expression up-regulated		
miR-18a-3p	miR-18a*	Apoptosis: *CASP7*, *KRAS*[#] Drug target: *CASP7* MAPK signalling pathway: *KRAS*[#] VEGF signalling pathway: *KRAS*[#]
miR-20a-5p	miR-20a	Angiogenesis: *CCND1*, *THBS1*[#] Apoptosis: *E2F1*[#], *TGFBR2*[#], *PTEN*[#] Cell cycle: *CCND1*, *E2F1*[#], *THBS1*[#] p53 signalling pathway: *CCND1*, *THBS1*[#], *PTEN*[#] VEGF-R signalling pathway: *BMPR2* Wnt signalling pathway: *CCND1* EMT: *TGFBR2*[#], *SMAD4*, *AKT3*, *TCF7L1*
miR-20a-3p	miR-20a*	Angiogenesis: *PPARA* Apoptosis: *KRAS* Cell cycle: *PCNA* DNA repair: *PCNA* GABAergic synapse: *SLC38A2* G-protein coupled receptor signalling pathway: *LPAR1* MAPK signalling pathway: *KRAS* VEGF signalling pathway: *KRAS*
miR-21-5p	miR-21	Angiogenesis: *MYC*, *TP53* Apoptosis: *APAF1*, *BTG2*, *E2F1*, *MYC*, *TP53*, *TGFBR2*[#], *PTEN*[#] Cell cycle: *TP53* Inflammation: *IL6R*, *TP53* MAPK signalling pathway: *TP53* p53 signalling pathway: *TP53*, *APAF1*, *PTEN*[#] VEGF-R signalling pathway: *BMPR2* Wnt signalling pathway: *TP53* EMT: *TGFBR2*[#]
miR-21-3p	miR-21*	Apoptosis: *CASP7*, *IKBKB*, *SMAD7*, *TGFB2* Cell cycle: *PAPD5*, *TGFB2* Cell migration: *TGFB2* Drug target: *CASP7* EMT: *TGFB2* Inflammation: *IKBKB* MAPK signalling pathway: *IKBKB*, *MAP3K1*, *TGFB2*
miR-22-5p	miR-22*	Cell adhesion: *PCDH15* Cell migration: *NEXN* MAPK signalling pathway: *MAP3K2* Protein transport: *VPS26B* Response to DNA damage stimulus: *UBR5* Ubiquitin mediated proteolysis: *UBR5*
miR-22-3p	miR-22	Activin receptor signalling pathway: *LEMD3* Apoptosis: *ESR1*, *E2F2*, *NR3C1* BMP signalling pathway: *BMP7*[#], *LEMD3* Cell cycle: *BMP7*[#], *CDK6*, *E2F2* Cell migration: *CDK6* EMT: *BMP7*[#] Endocytosis: *TFRC* Hedgehog signalling pathway: *BMP7*[#] Inflammation: *HDAC4* p53 signalling pathway: *CDK6* SMAD protein signal transduction: *BMP7*[#] TGF-beta signalling pathway: *BMP7*[#], *LEMD3*, *NR3C1*

Supplementary Table 2 continued:

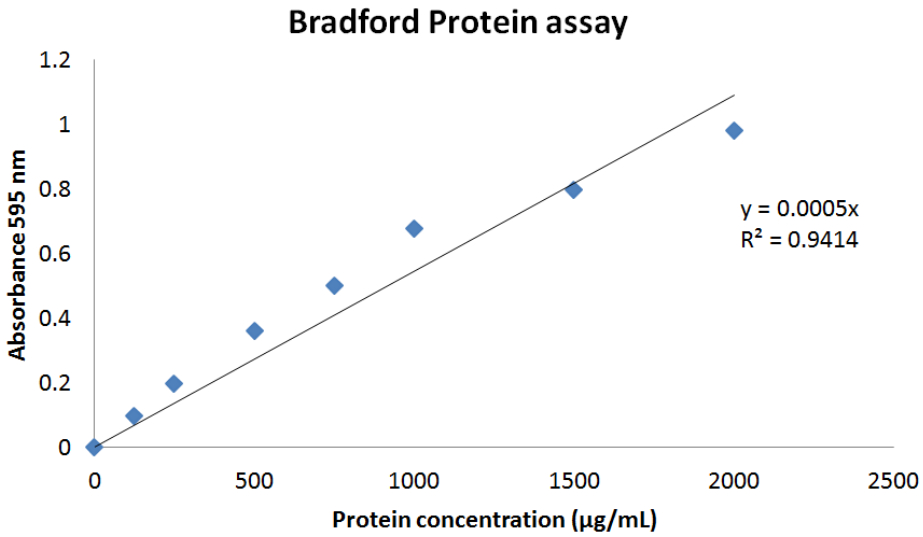
miRNA	Other name	Affected functions/processes: Selected target mRNAs
miRNA-5p/3p co-expression up-regulated		
miR-27a-5p	miR-27a*	Angiogenesis: *PDGFA* Apoptosis: *ATM, GSPT1* Cell cycle: *ATM, PRMT5* Histone methylation: *PRMT5* p53 signalling pathway: *ATM*
miR-27a-3p	miR-27a	Angiogenesis: *CCND1, CTSD, IGF1R, MET, TP53* Apoptosis: *ATM, FOXO1*[#]*, IGF1R, TP53, EGFR*[#] Cell cycle: *ATM, CCND1, PCNA, TP53* DNA repair: *PCNA* Focal adhesion: *MET* Inflammation: *TP53* MAPK signalling pathway: *TP53, EGFR*[#] p53 signalling pathway: *ATM, CCND1, TP53* Wnt signalling pathway: *CCND1, TP53*
miR-151a-5p	miR-151-5p	Apoptosis: *ARHGDIA* Drug metabolism – cytochrome P450/ metabolic pathways: *CYP2E1*
miR-151a-3p	miR-151-3p; miR-151	Angiogenesis: *EFNB2, THBS1* Cell cycle: *THBS1* EMT: *ZEB1* Transcriptional misregulation in cancer: *ZEB1* p53 signalling pathway: *THBS1*
miR-29b-1-5p	miR-29b-1*	Apoptosis: *BIRC2, PAK7, SIRT1, TCF7L2* Cell cycle: *CCNA2, LIN9, TCF7L2* Cell differentiation: *NR2C2* Cell migration: *PAK7* DNA methylation: *PRMT3, PRMT8* DNA repair: *SIRT1* EMT: *TCF7L2* Inflammation: *IL6ST, NR3C1*
miR-29b-3p	miR-29b	Apoptosis: *CASP7, PTEN* DNA methylation: *DNMT3A/B* Drug target: *CASP7* Inflammation: *HDAC4, SLC7A5* p53 signalling pathway: *PTEN*
miR-31-5p	miR-31	Angiogenesis: *ETS1, MYC, TIMP2, TP53* Apoptosis: *CDKN2D, MYC, TP53* Cell cycle: *CDKN2B, CDKN2D, TP53* DNA repair: *CDKN2D* Inflammation: *FOS, RHOA*[#]*, TP53* MAPK signalling pathway: *TP53* p53 signalling pathway: *TP53* Wnt signalling pathway: *TP53*
miR-31-3p	miR-31*	Apoptosis: *ACVR1, VDAC1* Inflammation: *ACVR1* Protein localisation/ transport: *VPS13A*
miR-199a-5p	miR-199a	Angiogenesis: *HIF1A, MET* Apoptosis: *CAV1*[#]*, IKBKB*[#] Cell cycle: *CDKN1B* Cell migration: *HIF1A* Focal adhesion: *CAV1*[#]*, MET* VEGF signalling pathway: *HIF1A* EMT: *GSK3B* MAPK signalling pathway: *IKBKB*[#]

Supplementary Table 2 continued:

miRNA	Other name	Affected functions/processes: Selected target mRNAs
miRNA-5p/3p co-expression down-regulated		
miR-199a-3p	miR-199a*	Angiogenesis: *AKT1, CD44, MET*[#] Apoptosis: *AKT1, MAPK1, MAPK8*[#] BMP signalling pathway: *ACVR2A* Cell migration: *AKT1* Focal adhesion: *AKT1, MAPK8*[#]*, MAPK9, MET*[#] Inflammation: *CD44* MAPK signalling pathway: *AKT1, MAPK1, MAPK8*[#]*, MAPK9, MAP3K4* Response to stress: *MAP3K4, MAPK8*[#]*, MAPK9* VEGF signalling pathway: *AKT1, MAPK1* Wnt signalling pathway: *MAPK8*[#]*, MAPK9*
miR-378a-5p	miR-378*	Angiogenesis: *PTGS1* Apoptosis: *BAG1, DCC, DFFA, FRZB, GABPA* DNA repair: *RAD1* Inflammation: *IL1R1* Response to oxidative stress: *SLC7A11*
miR-378a-3p	miR-378	Angiogenesis and tumour growth: *SUFU*[#] DNA repair: *MSH3*
miR-574-5p	miR-574-5p	Angiogenesis: *BMP4, NR4A2* DNA damage response: *FOXN3*[#] Inflammation: *BMP4* Transcriptional misregulation in cancer: *WHSC1*
miR-574-3p	miR-574	Cell cycle: *CHEK1, CKS2* DNA repair: *CHEK1* Drug target: *CDC25B* Histone methylation: *CARM1* p53 signalling pathway: *CHEK1*
miRNA-5p/3p inversed regulated		
let-7d-5p (up-regulated)	let-7d	Angiogenesis: *IGF1R*[#] Apoptosis: *IGF1R*[#] Cell cycle: *THBS1*[#] EMT: *HMGA2* p53 signalling pathway: *THBS1*[#]
let-7d-3p (down-regulated)	let-7d*	Apoptosis: *KRAS* G-protein regulatory: *PRKACB* MAPK signalling pathway: *KRAS*
miR-200b-5p (down-regulated)	miR-200b*	Chromatin modification: *PRDM6* EMT: *ZEB2* Small GTPase mediated signal transduction/GTP catabolic process: *RAB1A* Wnt signalling pathway: *ZEB2*
miR-200b-3p (up-regulated)	miR-200b	EMT: *ZEB1*[#]*, ZEB2* Mismatch repair: *MLH1* Transcriptional misregulation in cancer: *ZEB1*[#]

Hash (#) indicates validated target. Data derived from the miRNA target prediction algorithm (as described in Materials and Methods Section 3.6), NCBI Gene Ontology Annotation Database (UniProt-GOA) and KEGG Pathway databases.

Appendix C



Supplementary Figure 1: Protein lysates quantitation by Bradford protein assay. The equation y = 0.0005x was used to calculate the concentration of protein lysates prepared from the colon cancer cell lines.

Supplementary Table 3: Protein lysates quantitation by Bradford protein assay

Cell lines	HT-29	SK-CO-1	WiDr
Absorbance 595 nm	0.5547	0.4386	0.4847
Protein concentration (µg/mL)	11093.67	8771.67	9694.33
Protein yield (µg)	5546.83	4385.83	4847.17

Appendix D

Participated Conferences

Conference: The 10th Asia Pacific Conference on Human Genetics
Organiser: Asia Pacific Society of Human Genetics
Date/venue: 5-8 Dec 2012, Crowne Plaza Hotel, Kuala Lumpur, Malaysia.
Presented by: Soon Yuen Loon (Poster)

Global microRNA (miRNA) expression profiling in human colon cancer cells

Yuen Loon Soon[1, 2], Chiu-Jung Huang[3, 4], Chee Keat Loh[5], Kong Bung Choo[1, 2]

[1]Faculty of Medicine and Health Sciences & [2]Centre for Stem Cell Research, Universiti Tunku Abdul Rahman, Selangor, Malaysia;
[3]Department of Animal Sciences & [4]Institute of Biotechnology, Chinese Culture University, Taipei, Taiwan;
[5]WaferGen Biosystems (M) Sdn. Bhd., Kulim, Kedah, Malaysia

Introduction: miRNAs contribute significantly to post-transcriptional regulation of gene expression and are frequently dysregulated in cancers, including colon cancer.

Objective: The aim of this study was to establish the miRNA expression profiles in colon cancer cells.

Methodology: To identify miRNAs that are differentially expressed between normal colon tissues and colon cancer cells, four colon cancer cell lines (HCT-15, HT-29, SK-CO-1, WiDr) were subjected to miRNA profiling using a nanoliter-scale real-time PCR-based array (WaferGen Biosystems). Relevant information from the complex miRNA expression profiles in the cancer samples was extracted by bioinformatics tools and by statistical analyses. Real-time qPCR was performed on selected miRNAs to validate results from the array analysis. Targeted mRNA and protein levels in cancer cells were also determined.

Results: Of the 1,200 miRNAs included in the array, 128 miRNAs (10.6%) are found to be differentially expressed in colon cancer cells relative to cells in normal colon tissues. Among the 128 miRNAs, 92 are up-regulated and 36 are down-regulated in cancer cells, signifying alteration of miRNA expression in carcinogenesis. In hierarchical clustering analysis, some miRNAs are found to be expressed dissimilarly in different colon cancer cell types. We also found that numerous alternative miRNA* species are altered in colon cancer cells indicating biological significance. Further focuses on the miRNAs that are expressed similarly in all four cancer cell types will be presented and discussed.

Conclusion: A miRNA differential expression profile of colon cancer cells has been developed which may be used for further development of colon cancer-specific signature for diagnostic purposes.

Conference: Keystone Symposia: Noncoding RNAs in development and cancer
Organiser: Keystone Symposia
Date/venue: 20-25 Jan, 2013, Fairmont Hotel Vancouver, Vancouver, Canada
Presented by: Prof Dr Choo Kong Bung

Both the miRNA-5p and -3p strands are frequently co-expressed and are similarly dysregulated in colon cancer cells

Chiu-Jung Huang[1], Yuen Loon Soon[2], Kong-Bung Choo[2]

[1]Department of Animal Sciences, Chinese Culture University, Taipei, Taiwan;
[2]Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, Selangor, Malaysia

A miRNA gene may generate two mature miRNA species, namely the miR-5p and -3p species derived from the 5'- and 3'-strands of dicer-cleaved mature miRNA duplex. In most cases, only the 5p species remains while the 3p strand is degraded, or vice versa in fewer cases. However, recent studies have demonstrated frequent co-existence of the 5p and 3p strands both of which are biologically important. In this work, we examined the co-expression of miRNA alternative species in colon cancer cells to investigate possible their contributions to pathogenesis.

To identify differentially expressed miRNAs in colon cancer, four colon cancer cell lines (HCT-15, HT-29, SK-CO-1, WiDr) were subjected to miRNA expression profiling using a nanoliter-scale real-time PCR-based platform of WaferGen Biosystems. Of the 1,200 miRNAs included in the array, 128 miRNAs (10.6%) are differentially expressed in colon cancer cells relative to cells in normal colon tissues. Amongst the differentially expressed miRNAs, 92 are up-regulated and 36 are down-regulated in cancer cells, signifying significant alteration of miRNA expression profile in cancer cells. Randomly selected miRNAs were largely confirmed in real-time RT-PCR.

Furthermore, 19 miRNAs are found to be activated in cancer cells as they are beyond the threshold of detection in normal cells. Similarly, 12 miRNAs are shut-down in cancer cells since they are detected only in normal cells. Nineteen (19) pairs miRNAs which have either or both the 5p/3p species being significantly dysregulated were further identified in the cancer cells indicating frequent 5p/3p co-expression. The 5p/3p pairs are mostly either similarly up-regulated (14 pairs) or down-regulated (3 pairs) in cancer cells.

The target genes of 9 miRNA pairs that fall within three miRNA families are found to have different functions for the 5p and 3p species, supporting that miRNA alternative species, when co-expressed, may contribute differently to the tumorigenesis process. On analysis of target genes regulated by the miRNA pairs, previously unreported miRNAs in our list may be candidates that regulate genes in the apoptosis, EMT and metastasis pathways of tumorigenesis.