Exploring Long non-coding RNAs and Metabolic Proteins as Potential Biomarkers for Colorectal Cancer

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgment has been made in the text.

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Abstract

Colorectal cancer (CRC) has been ranked as the third most diagnosed malignancy and the second leading cause of cancer-related death. According to the World Health Organization, 1.8 million new cases, and 0.86 million deaths were recorded in 2018. Locally, the incidence of CRC continued to rise, and it became the most common cancer representing 17% of total cancer diagnosed and ~15% of total cancer-related death in 2017. The carcinogenesis of CRC starts from genetic or epigenetic mutation and goes through a cascade of regulatory signaling pathways such as epithelial-mesenchymal transition (EMT) autophagy, PI3K/AKT, MAPK, p53, and mTOR signaling resulting in the tumour.

Despite the implementation of screening programmes, and advanced treatment modalities, the outcomes are not satisfactory due to the development of metastasis/chemo-resistance, or the absence of predictive/prognostic biomarkers. The aim of this project was to explore epigenetic and metabolic factors as potential biomarkers for CRC. We hypothesize that long non-coding RNAs (lncRNAs) and specific metabolic proteins may be differentially expressed in CRC. The objectives are (i) to identify novel oncogenic autophagy-modulating lncRNAs in CRC cells and elucidate their molecular mechanism in carcinogenesis using an *in vitro* model; and (ii) to determine the expression of specific metabolic proteins in mouse xenograft CRC tumours that responded differentially after radiotherapy.

In the first study, we identified a group of differentially expressed autophagy-modulating lncRNAs in CRC cells after RNA-sequencing. Among the 32 isolated differentially expressed autophagy-modulating lncRNAs, we validated the most differentially expressed lncRNA, cancer susceptibility candidate 9 (CASC9) expression profiles and explored its molecular mechanisms in

CRCWe validated the expression of CASC9 in two publicly available datasets and the results showed that CASC9 is upregulated in CRC samples compared to their adjacent normal tissues, and higher CASC9 expressions reduced the overall survival of patients. We also demonstrated that the expression of CASC9 is higher in CRC cell lines (HCT-116, SW480, HT-29, and DLD-1) than normal colon cell line (CCD-112CoN). To evaluate the biological and physiological functions of CASC9 in CRC cells, we performed the Dicer-substrate mediated siRNA of CASC9 (Dsi-CASC9) gene silencing in HCT-116 and SW480 cells. The results indicated that effective and consistent silencing of CASC9 significantly reduced cell proliferation, migration, and colony formation in both cell lines. The key molecular pathways associated with carcinogenesis were explored using western blotting (WB). The results confirmed that the silencing of CASC9 significantly increased cellular self-degradation process autophagy. Besides, Dsi-CASC9 treated cells significantly phosphorylated the AMP-activated protein kinase (AMPK), downregulated protein kinase B (AKT) and the mammalian target of rapamycin (mTOR) in HCT-116 and SW480 cells. Furthermore, CASC9 silencing significantly alters the expression of EMT marker proteins E-cadherin, N-cadherin, and vimentin.

Using the same techniques above, we explored the role of a newly identified lncRNA called RNA associated with metastasis-11 (RAMS11). RAMS11 was reported to be upregulated in metastatic CRC and it promotes aggressive phenotype *in vitro* and *in vivo*. We are novel in exploring the molecular mechanisms and demonstrated that silencing of RAMS11 significantly promote autophagy and apoptosis, downregulate AKT/mTOR signaling via the AMPK pathway, and inhibit EMT markers and transcription factors in CRC cells. Both CASC9 and RAMS11 might be used as potential biomarkers and silencing these lncRNAs may be used for personalized CRC management.

To achieve the second objective of this study, we used the CRC tumours previously generated from a mouse xenograft model which received 15 Gy of irradiation and tumour size measured daily. The tumours were then collected and divided into unirradiated (control), poor responders and good responders based on their tumour size changes. We selected 3 samples from each group for high through-put proteomics analysis. Nine metabolic proteins which belong to some essential biochemical pathways such as glycolysis (PGK1, PGAM1, ENO1, PKM, TKT), ammonia detoxification (GLUD1), carcinogenesis (LDHA, GAPDH), and drug responses (MDH2) were being shortlisted. To warrant our findings, we further validated the expressions of PGK1, GLUD1, LDHA and GAPDH in the tumours by WB. Our results suggested that these proteins may be used as potential biomarkers for radiotherapy response in CRC.

Overall, this thesis demonstrated the oncogenic roles of CASC9 and RAMAS11 in CRC. We showed that CASC9 and RAMS11 were upregulated in CRC cell lines and silencing of these lncRNAs reduced CRC cells proliferation, viability, and migration through mTOR-dependent autophagy and EMT pathways. Both CASC9 and RAMS11 might be used as potential biomarkers. We also proposed 9 metabolic proteins as potential RT biomarkers. Further investigation is needed to translate our findings into clinical practice and enhance the treatment outcomes of CRC by personalized medicine and radiotherapy.

Publications

Papers in Academic Journals

Md Zahirul Islam Khan, Shing Yau Tam, and Helen Ka Wai LAW. Autophagy-Modulating Long Noncoding RNAs (LncRNAs) and Their Molecular Events in Cancer. Frontiers in Genetics, 2019; 9:750. (DOI: 10.3389/fgene.2018.00750).

Md Zahirul Islam Khan, and Helen Ka Wai LAW. Cancer Susceptibility Candidate 9 (CASC9) Promotes Colorectal Cancer Carcinogenesis via mTOR-Dependent Autophagy and Epithelial–Mesenchymal Transition Pathways. Frontiers in Molecular Biosciences, 8:627022. (DOI: 10.3389/fmolb.2021.627022)

Manuscript under Review

Md Zahirul Islam Khan, Helen Ka Wai LAW. RAMS11 promotes CRC through mTOR-dependent inhibition of autophagy, suppression of apoptosis, and promotion of epithelial-mesenchymal transition. Submitted to Cancer Cell International. (Manuscript ID: CCIN-D-21-00354R3).

Md Zahirul Islam Khan, Shing Yau Tam, Zulfikar AZAM, and Helen Ka Wai LAW. Proteomic profiling of metabolic proteins as potential biomarkers of radioresponsiveness for colorectal cancer. Submitted to Cancers. (Manuscript ID: cancers-1234121)

International Conferences

Md Zahirul ISLAM KHAN and Helen Ka Wai LAW. Autophagy Drugs are Dynamic Regulators of Long non-coding RNAs (lncRNAs) Expression in Colorectal Cancer (CRC). ESMO World Congress on GI Cancer, Virtual, 1-4 July 2020. (Abstract and Poster Presentation, Abstract ID # 306)

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Md Zahirul ISLAM KHAN and Helen Ka Wai LAW. The Role of Long non-coding RNA RAMS11 in Promoting Colorectal Cell Development and Metastasis. ESMO Targeted Anticancer Therapies Virtual Congress 2021. 1-2 March 2021. (Abstract and Poster Presentation, Abstract ID # 294). **The Recipient of Travel Grant Award.**

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List of acronyms

2D-PAGE	Two-dimensional gel electrophoresis
3DCRT	3-dimension conformation RT
5-FU	5-fluorouraicil
AJCC	American Joint Committee on Cancer
AMPK	AMP-activated protein kinase
ATCC	American Type Culture Collection
ATGs	Autophagy-related genes
BANCR	BRAF-activated long non-coding RNA
BDC	Bladder cancer
BRAF	B-raf gene
BRC	Breast cancer
BSA	Bovine serum albumin
BT	Brain tumour
CA	Carbohydrate antigen
CA19-9	Carbohydrate antigen 19-9
CASC2	Cancer susceptibility candidate gene 2
CASC9	Cancer susceptibility candidate gene 9
CCK-8	Cell counting Kit-8
CD152	Cluster of differentiation 152
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
circRNAs	Circular RNAs

COAD	Colon adenocarcinoma
CRC	Colorectal cancer
DEPs	Differentially expressed proteins
DFS	Diseases-free survival
DMEM	Dulbecco’s modified eagle medium
dMMR	Mismatched repair deficient
DMSO	Dimethyl sulfoxide
ECL	Electrochemiluminescence
EGFR	Epidermal growth factor receptor
EMA	European Medicinal Agency
EMEM	Eagle’s minimum essential medium
EMT	Epithelial-mesenchymal transition
ENCORI	The Encyclopedia of RNA Interactomes
ENDC	Endometrial cancer
ER+	Estrogen receptor
ESCC	Esophageal squamous cell carcinoma
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery rate
FOBT	Fecal occult blood test
GAS5	Growth arrest specific 5
GC	Gastric cancer
GEPIA	Gene Expression Profiling Interactive Analysis

GMEC	Gliomma microvascular endothelial cancer
GO	Gene ontology
GSEA	Gene Set Enrichment analysis
GWES	The genome-wide expression profiles
HCC	Hepatocellular carcinoma
HER2+	Human epidermal growth factor receptor 2
HGNC	HUGO Gene Nomenclature Committee
HMGB1	High-mobility group box 1
HNC	Head and neck cancer
HOTAIR	HOX transcript antisense RNA
HR	Hazard ratio
HR-	Hormone receptor
HRP	Horseradish peroxide
HULC	Highly up-regulated in liver cancer
iTRAQ	Isobaric tag for relative and absolute quantification
JAK	The Janus kinase/signal transducers
LC	Liquid chromatography
LCPAT1	Lung cancer associated transcript 1
LD-CT	Low-dose computed tomography
lncRNAs	Long non-coding RNAs
LNM	Lymphatic node metastasis
LUC	Lung cancer
LVC	Liver cancer

MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MALDI-TOF	Matrix-assisted laser desorption ionization time of flight
MEG3	Maternally expressed gene 3
miRNAs	Micro-RNAs
MM	Multiple myeloma
MPM	Malignant pleural mesothelioma
MS	Mass spectrometry
MSI-H	Microsatellite instability-high
mTOR	Mechanistic targets of rapamycin
NCI	National Cancer Institute
NCI	National Cancer Institute
ncRNAs	Non-coding RNAs
NGS	Next-generation sequencing
NPC	Nasopharyngeal carcinoma
NSCLC	Non-small cell lung cancer
OS	Overall survival
OSTS	Osteosarcoma
OVC	Ovarain cancer
PANDA	Promoter of CDKN1A antisense DNA damage activated RNA
PBS	Phosphate buffer
PCA3	Prostate cancer antigen 3
PD1	Programmed cell death 1
PDA	Pancreatic ductal adenocarcinoma

piRNAs	Piwi-interacting RNAs
PKM2	Pyruvate kinase isozymes 2
PNC	Pancreatic cancer
PPIB	Peptidylprolyl isomerase B
PPTC	Papillary thyroid carcinoma
PR+	Progesterone receptor
PRKD3	Protein kinase D3
PSA	Prostate-specific antigen
PTENP1	Pseudogene of tumor suppressor gene PTEN
PVT1	Plasmacytoma variant translocation 1
RAMS11	RNA associated with metastasis 11
RECIST	The Response Evaluation Criteria in Solid Tumour
RIN	RNA-integrity
ROR	The lncRNA regulator of reprogramming
RT	Radiation therapy
SELDI-TOF	Surface-enhanced laser desorption ionization-time of flight
SEM	Standard error of mean
shRNAs	Short hairpin RNAs
siRNAs	Short-interfering RNAs
SIRT1	Silent information regulator 1
snoRNAs	Small nucleolar RNAs
STAT3	Signal transducer and activator of transcription 3
SWATH	Sequential window acquisition of all theoretical fragment ion spectra

TCA	Tricarboxylic acid
TCGA	The Cancer Genome Atlas
TNM	Tumour node metastasis
TPM	Transcript per million
TRIM3	Tripartite motif-containing protein 3
TUG1	Taurine up-regulated 1
US-FDA	United States Food and Drug Administration
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Chapter 1

Background of research

1.1 The epidemiology of colorectal cancer (CRC)

CRC has been ranked as the third most diagnosed malignancy and the second leading cause of cancer-related death by GLOBOCAN statistics in 2018 (Bray et al., 2018, Bray et al., 2020). For the last few decades, the incidence and mortality was abundantly higher in western countries. However, the number of new cases and death steadily increasing in developing countries as well as East/Southeast Asia (Rawla et al., 2019). The formation of CRC was triggered by a sequences of genetic or epigenetic mutations of certain epithelial cells that acquired some selective advantages on their functional roles (Ewing et al., 2014). The abnormal cells then start to grow excessively and proliferate to form a benign adenoma which matured and turned into carcinoma, and lattermost metastasized to reach distant parts of the body through the bloodstream (Centelles, 2012).

According to World Health Organization (WHO), 1.8 million new cases and 0.86 million deaths were recorded in 2018 where 70% of cancer death were from low-and middle-income countries (World Health Organization, 2020). According to Hong Kong Cancer Registry, the rate of CRC incidence in Hong Kong is raising every year and it became the top most commonly diagnosed cancer in Hong Kong in 2017 (Hong Kong Cancer Registry, 2020). In 2017, there were 5,635 newly diagnosed CRC patients (17% of total cancer) and ~15% of death among total cancer-related death (Hong Kong Cancer Registry, 2020). Most of the CRC were diagnosed at age over 50 years and higher death is mostly due to the development of metastasis (Bray et al., 2018, Rawla et al., 2019). Globally, 20% of the patients diagnosed with CRC already had tumour metastasized to

distant parts of their body (Riihimäki et al., 2016, Bray et al., 2018). Cancer metastasis is the major cause of treatment failure.

1.2 Diagnosis of CRC

The current gold standard for CRC screening are colonoscopy and fecal occult blood test (FOBT). Colonoscopy has high sensitivity in detecting polyps or adenoma formed in the rectum wall and facilitate complete dissecting of polyps in a single session (Jeun et al., 2019). The patient compliance of colonoscopy is progressively reducing due to its high cost, invasiveness, and secondary complication such as perforation and bleeding associated with it (Jeun et al., 2019). Recently, the non-invasive FOBT screening approach is being promoted due to its simplicity and less cost. However, FOBT cannot be used to replace colonoscopy because it has relatively high false positive and false negative rates and unsatisfactory selectivity and sensitivity (Alves Martins et al., 2019).

The newer faecal immunochemical test (FIT) uses specific antibodies to detect human haemoglobin in faeces. It is reported that FIT is more sensitive compared to the traditional guaiac-based test and it is promoted by various health care organizations to facilitate the CRC screening process (Doubeni et al., 2016). The detection of human haemoglobulins rather than haem increased the specificity of the test (Cross et al., 2019). So that, FIT is now replacing the traditional FOBT or gFOBT in because of the higher uptake, self-usability and quantitative analysis with superior sensitivity in CRC screening (Cross et al., 2019).

1.3 Staging and molecular subtypes of CRC

1.3.1 Staging of CRC

The large intestine is consisted of two main parts; colon and rectum. Similar to other part of the intestines, the colon wall is made up of the inner mucosal layer, submucosal layer, muscle layer and the outer most layer called serosa (Bruening et al., 2014). During the progression of CRC tumour, a complex network of micronutrient delivery system will be established with lymph nodes and blood vessels around the tumour microenvironment. The staging of CRC primarily depends on the rate of augmentation of diseases throughout the various layers of the colon or its expansion to the other parts of the body (Arena and Bilchik, 2013, Bruening et al., 2014). The staging of CRC during the diagnosis is the predominant factor of its management because the success of CRC treatment is highly dependent on the current staging of the tumour (Arena and Bilchik, 2013, Bruening et al., 2014). The American Joint Committee on Cancer (AJCC) established a widely acceptable staging system “TNM” for CRC where T stands for tumour stage, N stands for nodal stages and M stands for metastasis (Bruening et al., 2014). Based on the TNM system, the overall CRC has been further divided into the following categories (Figure 1.1).

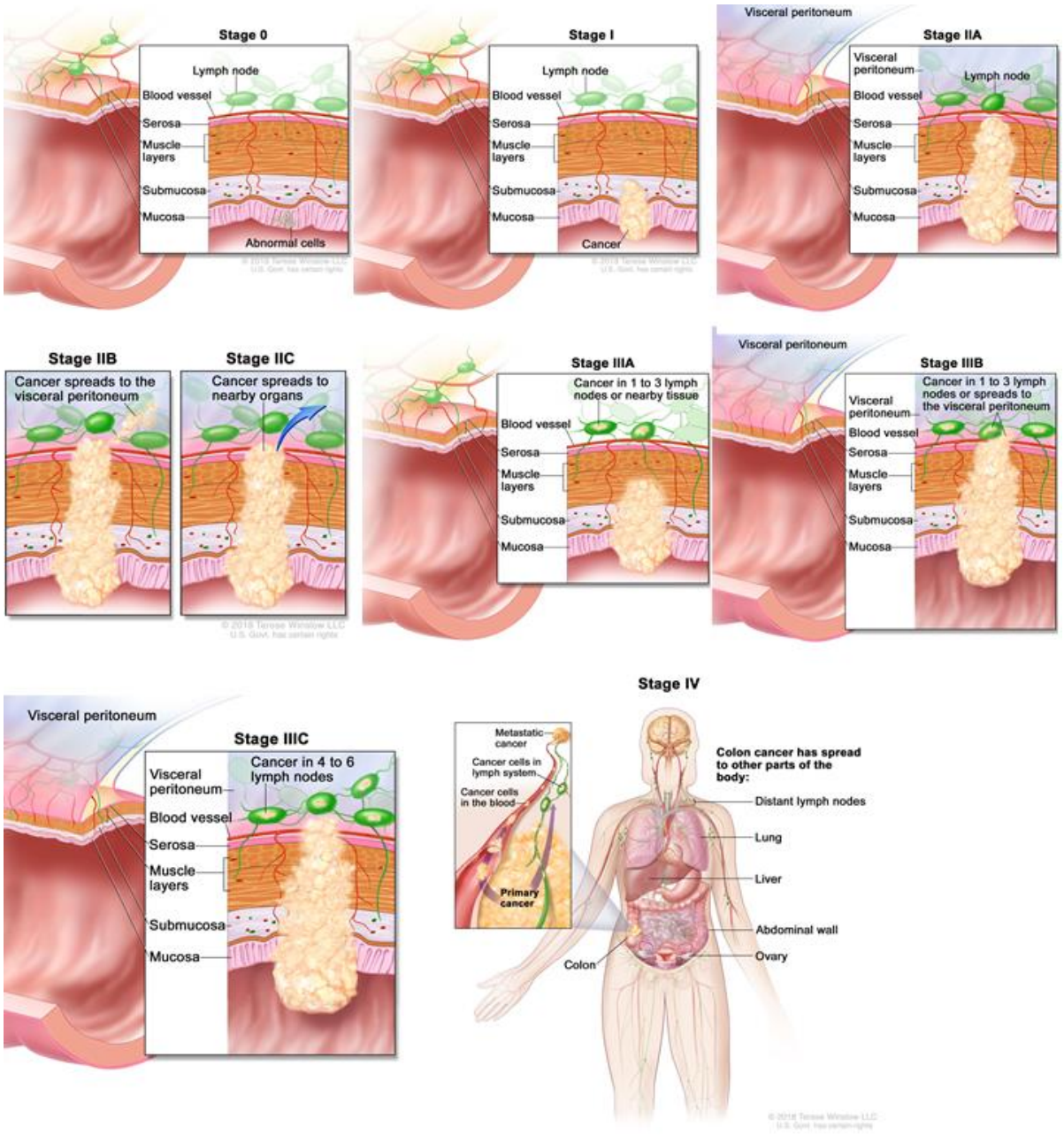


Figure 1.1. The stages of CRC. The stages of CRC categorised based on the TNM (T= tumour, N= Node, M= Metastasis). The severity of CRC increased with advanced stages of CRC. Each stages of CRC represents different TNM levels. Adopted from National Cancer Institute (National Cancer Institute, 2020).

Stage-0: Due to abnormal growth of cells, polyps usually formed into the inner layer of the colon which is called carcinoma in situ. Throughout this stage, cells lack spreadability, and there is no involvement of lymph nodes. They confined together in the inner wall of the rectum. Stage-0 CRC may also be considered as cancerous or precancerous stage. (Cancer Treatment Centers of America, 2020).

Stage-I: In stage-I CRC, the tumour enlarged to invade from inner mucosal layer into the submucosa or sometimes into the muscle layer. Up to this stage, there is still no lymph node involvement and there is no spread to other organs (Carethers, 2008).

Stage-II: Stage-II CRC penetrate into the abdominal cavity from its rectal origin but lack of lymph node involvement. Stage-II is further categorised into three parts:

- Stage-IIA: The tumour has grown through the outermost rectal layer serosa but no involvement of lymph nodes and no spreading to other organs (Bruening et al., 2014).
- Stage-IIB: The cancer has become more mature over the time and grown throughout the entire layer of rectum, still this stage, no lymph node involvement nor spreading to other organs (Cancer Treatment Centers of America, 2020).
- Stage-IIC: The tumour has grown throughout the intestinal wall and attached to the nearby organs or tissues such as the urinary bladder or uterus. But with no involvement of lymph nodes or distant organs (Cancer Treatment Centers of America, 2020).

Stage-III: At Stage-III CRC has started to spread to the nearest lymph nodes. This stage is sub-categorized into the followings:

- Stage-IIIA: The tumour has penetrated throughout all layers of rectum and may attach with up to 3 nearby lymph nodal connections (American Cancer Society, 2020).

- Stage-IIIB: The tumour matured and connected with nearby organs or tissues and may spread up to 6 nearby lymph nodes (American Cancer Society, 2020).
- Stage-IIIC: Up to this stage, CRC has not spread to any distant organs of the body but raised over the layer serosa and visceral peritoneum. At least 7 or more neighbouring lymph nodes are involved (American Cancer Society, 2020).

Stage-IV: Stage-IV is the most lethal and advanced stage of CRC. In Stage-IV, the abnormal cancer metastasized and spread to the distant part of the body (mostly livers and lungs). Based on the metastasis and its impact on various organ damage, it is further divided into two groups:

- Stage-IVA: The Stage-IVA refers to the spreading of cancer in just one organ such as only liver or lungs.
- Stage-IVB: On the other hand, when the cancer spreads to more than one organ it is called Stage-IVB (American Cancer Society, 2020, Cancer Treatment Centers of America, 2020).

1.3.2 Molecular subtypes of CRC

The desired and precise therapeutic outcome of CRC can be achieved dividing the patients based on their molecular subtypes. The application of "one drug fit all" is the current strategy for managing advanced stages CRC patients however, CRC is highly heterogeneous and each patient displays identical genetic and pathological signatures. So that, molecular subtypes is crucial for improving the therapeutic outcomes of individual patients (Singh et al., 2021). The clinical and molecular characteristics such as, microsatellite instability (MSI), mismatch repair system (MMR), and mutations in *APC, TP53, KRAS, BRAF, NRAS* with *PIK3CA* plays important roles in CRC pathogenesis. These are sometimes used as biomarkers in clinical settings for CRC management (Singh et al., 2021). However, the treatment outcomes of various therapeutic aspects differ

significantly based on the classification of CRC subtypes. Based on the candidate cancer gene (CAN), Sjoblom and team first introduced the term molecular subtypes of CRC and BRC since 2006 (Sjöblom et al., 2006). However, scientists established several other classification approaches in extent of genetic, epigenetic, gene expression profiling, and single cell analysis of the tumours. These CRC classification approaches largely differs from each other. So far, none of these classifications were not able to define the staging CRC clearly. These obscurity and differences between each subtype might be associated with methodological applications, different platform in generating data, and bias in statistical analysis (Sjöblom et al., 2006, Leary et al., 2008, De Sousa E Melo et al., 2013, Vogelstein et al., 2013).

The extent, heterogeneity, and nature of CRC pathogenesis are highly controlled by the molecular subtypes of the diseases. The molecular classification of CRC demonstrates the etiology and the characteristics of tumours that prompt us in better understanding and management of cancer (Coebergh van den Braak et al., 2020, Singh et al., 2021). However, most CRC subtypes are based on tumor stage, genetic description, cellular characteristics, cancer microenvironment, and immunological descriptions. Each classification approach provides an identical benefit to the clinical outcomes of the patients. The most common molecular subtypes are Jass classification (Jass, 2007), Ogino classification (Ogino and Goel, 2008), Colon cancer subtypes (De Sousa E Melo et al., 2013), Sadanandam CRC assigner system (Sadanandam et al., 2013), Colon cancer molecular subtype (Marisa et al., 2013), CRC intrinsic subtypes (Roepman et al., 2014), and Consensus molecular subtypes (Guinney et al., 2015).

1.4 Treatments of CRC

The management of CRC greatly depends on several factors including, proper diagnosis, size of tumour, extent of malignancy, invasiveness of the polyps and clinician decisions (Daaboul and El-Sibai, 2017). Still, surgery is the primary treatment option for Stage-I/II CRC patients while adjuvant therapy is sometimes applied to advanced Stage II CRC patients. The surgical techniques may vary depending of the size and depth of the tumours. For Stage-III CRC patients, surgery and followed by chemotherapy and/or radiation therapy (RT) usually recommended to improve the surgical outcomes of the patients. However, Stage-IV CRC is generally difficult to remove completely as it has metastasized to distant parts of the body. To manage the Stage-IV CRC, a combination of surgery, booster chemotherapy, RT and sometimes precision medicines are recommended to improve the overall survival (OS) and disease-free survival (DFS) of patients (Van Cutsem et al., 2013, Vogel et al., 2017, Kim and Kim, 2020). Based on the therapeutic options, benefits, probable risk factors, and considering side effects, treatment options are divided into local and systemic treatments.

1.4.1 Local treatments

The local treatment means the removal of tumour from the colon or rectum without damaging the remaining parts of the body. Local treatments are very effective against primary stages of CRC. The local treatment approach has improved remarkably in the last two decades along with surgical techniques and RT techniques and its application to multimodality courses (Geisler, 2007). The local treatments mainly consist of surgery, RT, and ablation and embolization.

1.4.1.1 Surgery

Preceding to localized surgery, patients should be carefully diagnosed to determine the appropriate staging of CRC (Mutch, 2016). In the planning stage, it has been suggested to assess carcinoembryonic antigen (CEA) level prior to surgery. However, there are still some controversy with the specificity and sensitivity of CEA as a diagnostic marker (Mutch, 2016). There are two main surgical approaches to CRC surgery including open surgery and minimally invasive surgery. The advanced neoplasia and early stages cancers can be removed endoscopically using endoscopic mucosal resection or endoscopic submucosal dissection, whereas surgery can be done to remove advanced stages tumours. Surgical removal of CRC may involve removing the polyps only, part of the colon, and sporadically removal of cancerous tissues with its adjacent normal tissues and neighbours lymph nodes (Cho et al., 2007). The adjuvant therapies including RT or chemotherapy are given to eradicate cancer cells and reduce the chance of cancer recurrence. The rate of success after primary tumour dissection is very high. However, bleeding or urinary tract obstruction are common side effects associated with it. The long-term management of CRC is strongly subjected to TNM stages, successful resection of tumours, and the use of adjuvant chemotherapies (Li et al., 2009, Mutch, 2016).

1.4.1.2 Radiation therapy (RT)

CRC patients with localized tumours are usually treated with RT along with chemotherapy or surgical excision (Glynne-Jones and Kronfli, 2011). RT significantly boost up treatment outcomes of surgery or chemotherapy by extending OS and DFS (Hatcher and Kumar, 2014). In advanced stages of completely inoperable CRC, RT is commonly prescribed with chemotherapy together (chemoradiotherapy) to effectively reduce tumour burden (Pezner et al., 1999). Palliative RT is also effective in relieving the secondary symptoms and the survival of patients with metastasis

(Barrett et al., 2009). Special techniques of RT for Stage-IV inoperable and elderly patients have been developed over the time which enhanced the prognosis and control of CRC (Tam and Wu, 2019). However, RT has side effects such as fatigue, urinary tract disorders, hair loss, and skin irritation are prominent while treating with extended chemotherapy (Pezner et al., 1999).

Referring to the oncological perspective, the anatomical dissimilation between colon and rectum is very crucial in applying RT. The rectum is part of large intestine comprising of peritoneal reflection to the anus (Saltz and Minsky, 2002, Häfner and Debus, 2016). The upper margin lies to the third sacral vertebra level which is about 13 cm in length (McDonald, 1993). The tumours above the reflection are commonly considered as colon and managed them in colonic paradigm. On the other hand, the adjuvant and neoadjuvant therapies are commonly applied to manage the rectal cancer (Lidder and Hosie, 2005, Tseng et al., 2018, Tam and Wu, 2019).

1.4.1.3 Ablation and embolization

Ablation techniques are useful for killing the tumours without dissecting them by surgery. Ablation and embolization are excellent substitutes for the management of metastatic CRC, especially for the recurrent tumours after surgery, inoperable tumours, and unfit for surgery due to other complications (Clark and Smith, 2014, Seidensticker et al., 2018). This therapy enhanced the patient survival and quality of life. The common techniques of ablation include radiofrequency ablation, microwave ablation, ethanol ablation, and cryo-ablation. Radiofrequency ablation is the most common technique where the distant tumours in the liver are treated by using a high-energy radio waves through a needle like probe into the tumours (Leggett et al., 2014). Similarly, in microwave ablation method, high energy electromagnetic microwaves applied to the tumours by the guidance of a needle like probe (Lubner et al., 2010). Alternatively, high concentration alcohol could be percutaneously injected to destroy the tumours in ethanol ablation methods. Very cold

gas may also be used to destroy tumours in cryo-ablation techniques (Knavel and Brace, 2013). Similar to the ablation techniques, a number of embolization methods are also used to destroy the tumours without surgery such as arterial embolization, chemoembolization, and radio-embolization.

1.4.2 Systematic treatments

The systemic treatments refers to the management of CRC by applying one or combined doses of drugs orally or intravenously. Drugs systematically reached to the cancer cells and effectively destroy them. Based on the different stages of CRC, there are different types of systematic treatments available, such as chemotherapy, immunotherapy, and targeted therapy.

1.4.2.1 Chemotherapy

In chemotherapy, specific drugs are used to destroy the cancer cells via a variety of routes like, oral administration, intravenous administration, and through infusion pumps (De Falco et al., 2020). The types of drugs and their administration methods are highly dependent on the type, stage, and metastatic nature of the cancer (Dekker et al., 2019). Palliative chemotherapy is prescribed in advanced stage CRC when it has already spread to distant part of the body. In metastatic CRC, chemotherapy is given to reduce the tumour size, alleviate primary and secondary symptoms, and enhance the patient survival (Mende et al., 2013). Adjuvant chemotherapy is mostly given as a follow-up treatment after the dissection of tumours. Adjuvant chemotherapy effectively kills the remaining tumour cells (Kountourakis et al., 2016). Alternatively, neoadjuvant chemotherapy is administered prior to the surgery that improve surgical outcomes and reduce the secondary complications related to the surgery (de Gooyer et al., 2020).

The most commonly used chemotherapy drugs for CRC patients include 5-fluorouraicil (5-FU), capecitabine, ironotecan, oxaliplatin, and a combination of trifluridine and tipiracil. Intravenous 5-FU has been traditionally used and is still the first choice of drug for CRC (Pardini et al., 2011). To enhance the effectiveness of 5-FU, intramuscular leucovorin calcium (folinic acid) is administered to minimize the secondary adverse effects caused by methotrexate, pyrimethamine, methanol poisoning, and anaemia (Wang et al., 2017b). Capecitabine, a pro-drug of 5-FU, may also be given to CRC patients after RT or surgery to act as adjuvant or neo-adjuvant therapy (Wang et al., 2014a). Recent chemotherapy development introduced numerous chemo-drugs such as aflibercept, bevacizumab, and ramucirumab which are mostly prescribed as combined therapy with 5-FU to treat advanced stage CRC (Xie et al., 2020).

1.4.2.2 Immunotherapy

Immunotherapy has potential roles in anti-cancer initiation as well as suppression. In this therapy, the body's own defence system is being activated or deactivated to recognize and effectively eliminate harmful cells in the body. It is a relatively novel approach in cancer management and many are still under clinical trials (Ganesh et al., 2019). The immunotherapy approach is well recognized for successful treatment of melanoma. Then, the rapid establishment and its advancement considered them to be used in other types of solid tumours specially CRC (Ganesh et al., 2019). Recently, programmed cell death 1 (PD1)-blocking antibodies, pembrolizumab and nivolumab, have shown potential outcomes in inoperable or dissectible metastatic microsatellite instability-high (MSI-H) and mismatched repair deficient (dMMR) CRC as approved by United States Food and Drug Administration (US-FDA) (Yaghoubi et al., 2019). CTLA-4 or cluster of differentiation 152 (CD152) blocking monoclonal antibody ipilimumab, has also shown potential immune turnover abilities to mediate killing of cancer cells (Morse et al., 2019). Intravenous

infusion of ipilimumab is mostly recommended to be administered along with other cancer drugs such as nivolumab to enhance the prognosis of CRC (Gourd, 2018).

1.4.2.3 Targeted therapy

Despite the recent advancement of CRC management, advanced stages of CRC are still poorly treated. However, improved understanding of molecular pathways associated with cancer cell growth and proliferation has led to the development of personal therapy, which selectively inhibit the cancer by acting on those pathways (Piawah and Venook, 2019). The approach of personal therapy is more specific and act differentially from other types of treatments. It can be used when chemotherapy or RT failed to treat the cancer or may use along with chemotherapy to significantly enhance the survival of patients (Willett et al., 2007). The successful development of targeted therapy primarily activating/inhibiting pathways including, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), B-raf gene (BRAF), and other targets (Lee and Oh, 2016).

VEGF and EGFR are upregulated in CRC and are associated with tumour stage and treatment outcomes. VEGF and EGFR promote cellular growth, proliferation, migration and leading to metastasis through the formation of new blood vessel network (angiogenesis) (Piawah and Venook, 2019). The monoclonal antibody bevacizumab and ramucirumab, and recombinant fusion antibody Ziv-aflibercept are approved by US-FDA and European Medicinal Agency (EMA) as these VEGF targeting drugs effectively inhibits angiogenesis-mediated CRC growth and prolong patients survival (Seeber and Gastl, 2016). Similarly, intravenous infusion of cetuximab and panitumumab monoclonal antibodies are also US-FDA and EMA approved. They are effective EGFR targeting drugs for treating various cancers including CRC (Seeber and Gastl, 2016). The efficacy and efficiency of anti-EGFR drugs are comparatively higher than anti-VEGF but mutations on KRAS

and BRAF genes are predetermining factors of treatment outcomes. Therefore, mutation of these genes must be identified prior to the prescription of anti-EGFR drugs. Alternative, the use of encorafenib (BRAF vanquisher) can be given together with cetuximab to improve treatment outcomes of CRC with any mutations (Lee and Oh, 2016, Seeber and Gastl, 2016, Piawah and Venook, 2019).

Regorafenib is another clinically approved multiple kinase-inhibiting drug, typically used to treat advanced metastatic CRC when other drugs failed to control the cancer (Piawah and Venook, 2019, Xie et al., 2020). There are abundant treatment options for CRC management. However, treatment outcomes still have rooms for improvement and many CRC research groups are trying to established early diagnostic and prognostic biomarkers, and novel therapeutic target to reduce CRC risk and improve prognosis worldwide.

1.5 Cancer biomarkers

The management of cancer predominantly depends on early diagnosis, proper staging, and suitable selection of treatment. More recently, research have been driven to identify biomarkers from patient's serum and tissues for diagnosis, prognosis, and therapeutic purpose. The DNA, mRNA, micro-RNAs (miRNAs), proteins, exosomes, enzymes, and metabolites are commonly considered as cancer biomarkers (Figure 1.2). The source of these biomarkers are not limited to the tumour itself or the blood but the exploration has been extended to other body fluids, neighbouring tissues and distant metastasis (Scatena, 2015).

The cancer biomarkers are commonly divided into 4 subtypes including diagnostic, prognostic, predictive and therapeutic biomarkers. Diagnostic biomarkers facilitate the early detection of cancer whereas prognostic markers may be used to access the prognosis of the disease, such as survival. Predictive biomarkers, similar to the prognostic markers, allow prediction of the clinical

outcome or recurrence of a disease. For therapeutic markers, they allowed us to evaluate the efficacy of the treatment (Italiano, 2011, Lin et al., 2012, Durães et al., 2014). For example, tumour specific molecular and histopathological characteristics, such as genetic mutation, alteration in DNA methylation, protein expression changes, dysregulation of non-coding RNAs expressions and presents of circulating tumour cells in bloodstream, are measured before treatment and be used as prognostic biomarkers (Pezo and Bedard, 2015, Carlomagno et al., 2017). On the other hand, some genetic materials (i.e., proteins, mRNA, RNAs) are commonly studied as therapeutic biomarkers for the design of targeted cancer therapy (Lin et al., 2012).

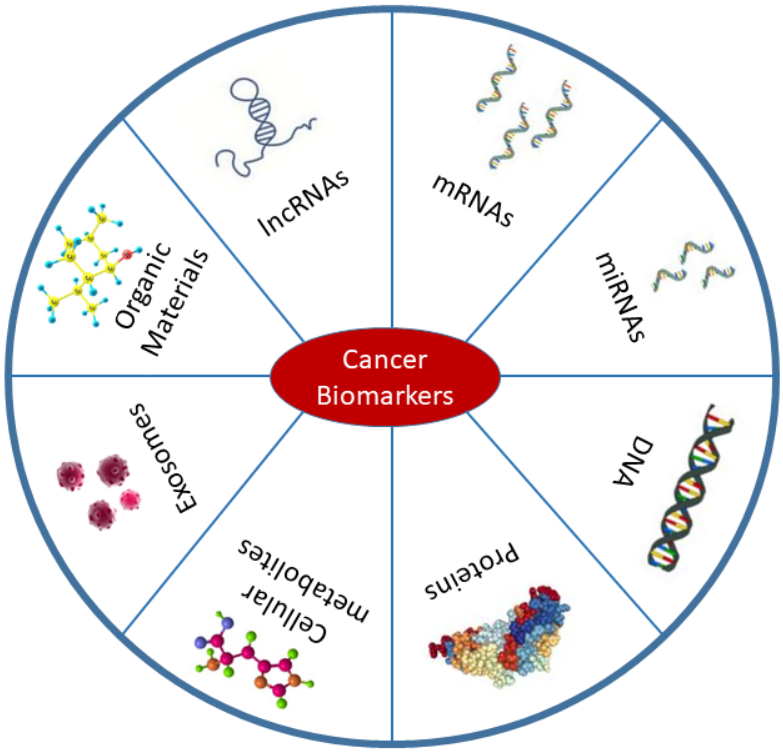


Figure 1.2. Types of various efficient cancer biomarkers. Biomarkers are mostly founded in body fluids including blood, urine, saliva, and cancer tissues. Cancer biomarkers belong to a wide range of biological elements such as DNA, mRNAs, proteins, long non-coding RNAs (lncRNAs), miRNAs, exosomes, cellular metabolites, and organic materials. This figure was based on published article (Wu and Qu, 2015).

According to the definition of National Cancer Institute (NCI), cancer biomarkers are biological molecules derived from our body which are differentially expressed or affected in carcinogenesis compared to the normal state (Henry and Hayes, 2012). The screening and identification of potential biomarkers depends on many factors such as type of cancer, associated microenvironment, metabolic nature of the tumour, and associated body responses. Nowadays, the advance knowledge and technologies in next-generation sequencing (NGS), gene expression arrays and high-throughput mass spectroscopy have allowed prompt identification of biomarkers for individual types of cancer (Chatterjee and Zetter, 2005, Henry and Hayes, 2012, Scatena, 2015). These advance techniques are generating many big databases. The potential danger is that unsupervised data mining and screening without clear intention may lead to reporting of false positive biomarkers (Henry and Hayes, 2012, Scatena, 2015). In spite of recent progress in the development and screening of cancer biomarkers, there are still many misinformation to be addressed. To fill this gap, gene expression profile must be studied carefully to establish novel non-invasive, specific, and sensitive biomarkers for cancer diagnosis, prognosis and management (Alves Martins et al., 2019).

1.5.1 LncRNAs as cancer biomarkers

1.5.1.1 Introduction to non-coding RNAs (ncRNAs)

Human genomic research using high-throughput NGS technologies revealed very few and inconsiderable amounts of protein-coding regions in the humans genome. Although predominant parts of RNA are transcribed, very small amount ($\leqslant$ 2%) are capable of producing proteins (Birney et al., 2007). The remaining major part (>90%) of the human genome is therefore considered as ncRNAs. Initially ncRNAs were thought to be non-functional junk molecules. The advancement of sequencing and bioinformatics analysis have now identified more and more ncRNAs with

potential biological functions (Cipolla et al., 2018). It has become increasingly ticklish to identify ncRNAs functions but the cumulative evidence has confirmed that physiological and pathological changes are highly regulated by ncRNAs. Therefore, various human diseases like cancer, metabolic and neurodegenerative diseases are highly controlled by ncRNAs (Peng and Calin, 2018). By the advancement of genomic study, researchers discovered several groups of ncRNAs such as miRNAs, circular RNAs (circRNAs), and lncRNAs (Anastasiadou et al., 2017, Yamamura et al., 2017).

1.5.1.2 Types of ncRNAs

The ncRNAs are mainly divided into two groups according to their length: (i) short ncRNAs, that contains lower than 200 nucleotide, and (ii) lncRNAs that contains higher than 200 nucleotides. Short ncRNAs are further categorized into small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), short-interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), and miRNAs (Pian et al., 2016, Sherstyuk et al., 2018) whereas lncRNAs have been divided into 6 groups according to the nearby protein-coding genes (Thum and Condorelli, 2015, Uchida and Dimmeler, 2015, Wang et al., 2018a):

1. Sense lncRNAs: They are transcribed from the plus strand of protein coding transcripts and cover with exons by sharing the same promoter.
2. Antisense lncRNAs: They are transcribed from the negative strand of protein coding transcripts.
3. Intronic lncRNAs: They are transcribed from the intronic regions of a gene.
4. Intergenic lncRNAs: They are from the intermediate space of two protein coding genes. They are also called long intergenic long non-coding RNAs (lincRNAs).

5. Bidirectional lncRNAs: They are transcribed by sharing promoter of two opposite directions.
6. Circular lncRNAs: They are transcribed from splicing of a protein-coding genes and covalently enclosed as circular RNAs (ciRNAs).

1.5.1.3 Functional classification of lncRNAs

Although, most of the lncRNAs are commonly transcribed by RNA Polymerase-II, their biological mode of actions are very diverse and cell specific. Based on the execution of biological functions, lncRNAs are further categorized into four groups, namely: Signal, Decoy, Guide and Scaffold lncRNA (Figure 1.3) (Thum and Condorelli, 2015, Bhan et al., 2017, Huang et al., 2017b).

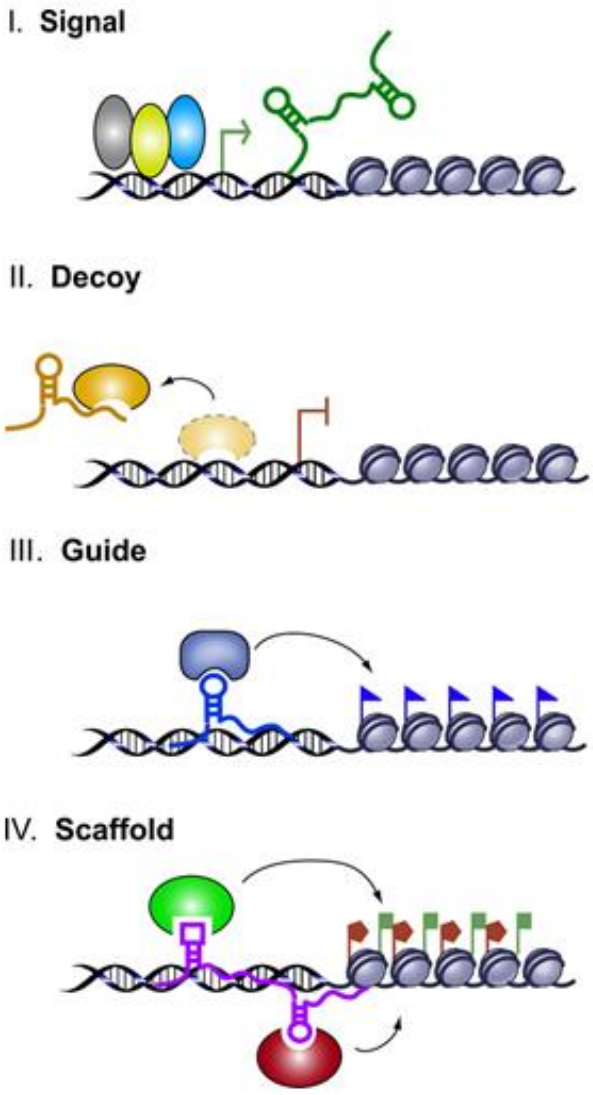


Figure 1.3. The functional classification of lncRNAs. (I) Signal: These signal lncRNAs are associated with specific expressions or pathways which can promote transcription regulated signaling events. (II) Decoy: These decoy lncRNAs can activate or silence genes and titrate away the transcription factors and miRNAs from chromatin. (III) Guide: Guide lncRNAs recruits ribonucleoprotein complexes to target genes either cis or trans form to regulate downstream signalings. (IV) Scaffold: These lncRNAs have multiple binding domains that can bind with numerous genes and direct them to the targeted genes. Adopted from (Wang and Chang, 2011).

Signal lncRNA: They are associated with cellular specific expression and specific pathways. They activate signaling events under particular transcriptional control. For example, linc-p21, PANDA (promoter of CDKN1A antisense DNA damage activated RNA) and TUG1 (taurine upregulated 1) are activate in DNA damage by tumor suppressor protein p53 (Chen et al., 2014).

Decoy lncRNA: These are the molecular sink of transcriptional factors and repressors. They activate or silence genes and titrate away the regulatory factors including transcriptional factors, catalyst, miRNA and chromatin modifier. For example, GAS5 (growth arrest specific 5) binds with the domain of DNA and glucocorticoids and titrate away from DNA response elements (Kino et al., 2010).

Guide lncRNA: Guide lncRNAs bind with regulatory ribonucleoprotein complexes and prescribe them to specific target genes to regulate downstream signaling events. For example, HOTAIR (HOX transcript antisense RNA) are prescribed in chromatin modifying complex (Hajjari and Salavaty, 2015).

Scaffold lncRNA: Scaffolds lncRNAs act as the central platform of lncRNAs. They have multiple binding domains that allow them to bind with multiple genes to form complex and direct them targeted genes which act as transcriptional activator or suppressors. For example, scaffolds lncRNA HOTAIR act as the bridge between complexes of HOTAIR/PRC2/LSD1 (Tsai et al., 2010).

1.5.1.4 LncRNAs in carcinogenesis

Among various kind of ncRNAs, the novel and rapidly emerging lncRNAs are ranked at high priority by researchers because of their involvement in diverse molecular events such as, transcriptional regulator, nuclear regions organization, and control of proteins molecules (Ulitsky

and Bartel, 2013). Modern research revealed that lncRNAs work as promoters of other RNAs from both sense and antisense strands to overlap genes, encode small proteins, and sometimes even act as small genes (Matsumoto et al., 2017, Kopp and Mendell, 2018).

LncRNAs are abundantly expressed in most cancers to alter normal biological processes. Their diverse expressions and mutations are inheritably connected with tumorigenesis, metastasis, and different tumor stages (Chan and Tay, 2018, Cipolla et al., 2018, Krishnan and Damaraju, 2018). The abundant expressions of lncRNAs from malignant tumors can be detected from circulatory blood or urine samples (Shi et al., 2016, Huang et al., 2018b). Thus, lncRNAs extended its application as discovery of potential biomarkers and therapeutic indicators of cancer to improve treatment outcomes. Herein below, we have summarized the roles of commonly studied lncRNAs and their potential applications in various cancer progression, metastasis, and in drug resistance.

The field of targeted therapy is rapidly developing with advanced genetic study and their successful applications. Previously considered junk molecules such as lncRNAs are now extensively studied to be established as novel diagnostic biomarkers and therapeutic targets. LncRNAs are associated with cancer cell proliferation, epigenetic modification, chromosome instability (Bolha et al., 2017). The abnormal expressions of lncRNAs are associated with the development and progression of most cancers. Therefore, this makes them important biomarkers for the diagnosis and prognosis of cancer. Recent studies revealed that lncRNAs are associated with epigenetic modification of various cancer types and take part in post translational modification of cancer cells (Sarfi et al., 2019). Gene expression alteration in carcinogenesis may starts with the binding of lncRNA with chromatin remodelling complex in either the same chromosomal or both chromosomal allele (Fatica and Bozzoni, 2014). In this approach, inherited gene expressions alteration takes place by recruiting chromatin modification factors (Martin and

Zhang, 2007, Lai and Shiekhattar, 2014). For example, HOTAIR is one of the first reported lncRNA associated with tumour progression via genome-wide epigenetic modification (Rinn et al., 2007, Gupta et al., 2010).

The advantage of using lncRNAs as potential diagnostic and prognostic biomarkers for cancer is due to their availability in circulating body fluids (Akers et al., 2013). A considerable number of studies have demonstrated that lncRNAs expressed in body fluid along with ribonucleases which can resist ribonuclease activities in cells (Shi et al., 2016). Likewise, dysregulation of lncRNAs in carcinogenesis can also be detected from wide ranges of body fluids, such as, blood, serum, gastric juice, and urine (Reis and Verjovski-Almeida, 2012, Sartori and Chan, 2014, Shao et al., 2014). These characteristics of lncRNAs have established them as effective, convenient, and minimally invasive biomarkers that compared with the conventional biopsies and invasive methods (Silva et al., 2015). Several lncRNAs have been studied in cancer individually or combinedly to compare the diagnostic accuracy compared with conventional biomarkers. As an example, MALAT1 have been proposed as a potential prognostic biomarkers in Stage-I non-small cell lung cancer (NSCLC) (Ji et al., 2003a). Another highly sensitive lncRNA, PCA3, have been identified in prostate cancer (PRC) urine sample. The sensitivity and specificity have shown better performance compared to the conventional PRC biomarker prostate specific antigen (PSA) (Fradet et al., 2004, Tinzl et al., 2004, Shappell, 2008). Similarly, RP11-445H22.4 overexpression was found to be in breast cancer (BRC) tissues and can be identified from serum samples with a specificity and sensitivity of 92%, and 74% respectively. This accuracy is better than the conventional BRC biomarkers CEA, CA125, and CA125 (Rasool et al., 2016). Similarly, HOTAIR, HULC, MALAT1, H19, MEG3, BANCR, and LINC00152 have been identified from a variety of biological fluids and demonstrated that they might be used as diagnostic, prognostic, and therapeutic targets for the management of various

cancer types (Jiang et al., 2016, Bolha et al., 2017, Li et al., 2019, Sarfi et al., 2019, Yu et al., 2020b).

Some lncRNAs have been identified from CRC samples and be proposed as CRC biomarkers. Study revealed that HOTAIR is upregulated in blood level and associated with poor prognosis and reduced OS of CRC patients (Svoboda et al., 2014). The diagnostic performance of HOTAIR shown 67% sensitivity, 92.5% specificity, and 0.87 area under curve value which proposed HOTAIR could be a potential diagnostic, prognostic, and therapeutic target for CRC management. Another study demonstrated that hepatocellular carcinoma (HCC) is associated lncRNA HANR. HANR is also significantly upregulated in CRC tumour samples and the higher expression can differentiate the normal tissues from carcinogenic tissues. Therefore, it has been suggested as a promising diagnostic and prognostic biomarker in CRC patients (Xu et al., 2020). Shen and colleagues also demonstrated that DANCR is highly upregulated in CRC patients serum which may enhance the diagnostic efficacy and monitor tumours together with CEA and carbohydrate antigen 19-9 (CA19-9) (Shen et al., 2020).

1.5.2 Autophagy-modulating lncRNAs as cancer biomarkers

Our team has strong interest in autophagy and lncRNAs. Hence we have reviewed the role of autophagy-modulating lncRNAs in a recent publication (Islam Khan et al., 2019). Together with some updates, the relevant contents are included in the following sections.

1.5.2.1 Introduction to autophagy

Autophagy is a highly conserved and critical regulatory process for cells to maintain homeostasis by lysosomal degradation of various proteins and damaged organelles. Dynamic roles of autophagy have been identified in cancers where it participates in cancer progression, prevention,

as well as, drug resistance mechanisms (Santana-Codina et al., 2017). There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy is sometimes also referred to as autophagy which is the major autophagic pathways and most extensively studied compared to microautophagy and chaperone-mediated autophagy. In macroautophagy, phagophore is initially formed and matured to autophagosome. Subsequently, autophagosome fused with a lysosome to degrade the internal materials in autolysosome (Figure 1.4) (Mizushima and Komatsu, 2011). Although autophagy may suppress tumours (Kung et al., 2011) in most cases, the induction of autophagy promotes tumorigenesis by improving survival capability of tumor under microenvironmental stress (Kung et al., 2011, Avalos et al., 2014, White, 2015). Autophagy promotes cancer by inhibiting tumor suppressor protein p53 and controlling the metabolism of cells (Amaravadi et al., 2016). Cellular metabolism and homeostasis are encoded by more than 30 autophagy-related genes (ATGs), their translational products and transduction of signals (Figure 1.4) (Kim and Lee, 2014, Cicchini et al., 2015, Ktistakis and Tooze, 2016). Tumorigenesis of both benign and malignant tumors are controlled by either single or group of ATG genes (Blessing et al., 2017, Mowers et al., 2017). Thus, in most of the cancers, autophagy is one of the therapeutic targets in clinical trials (Mowers et al., 2016).

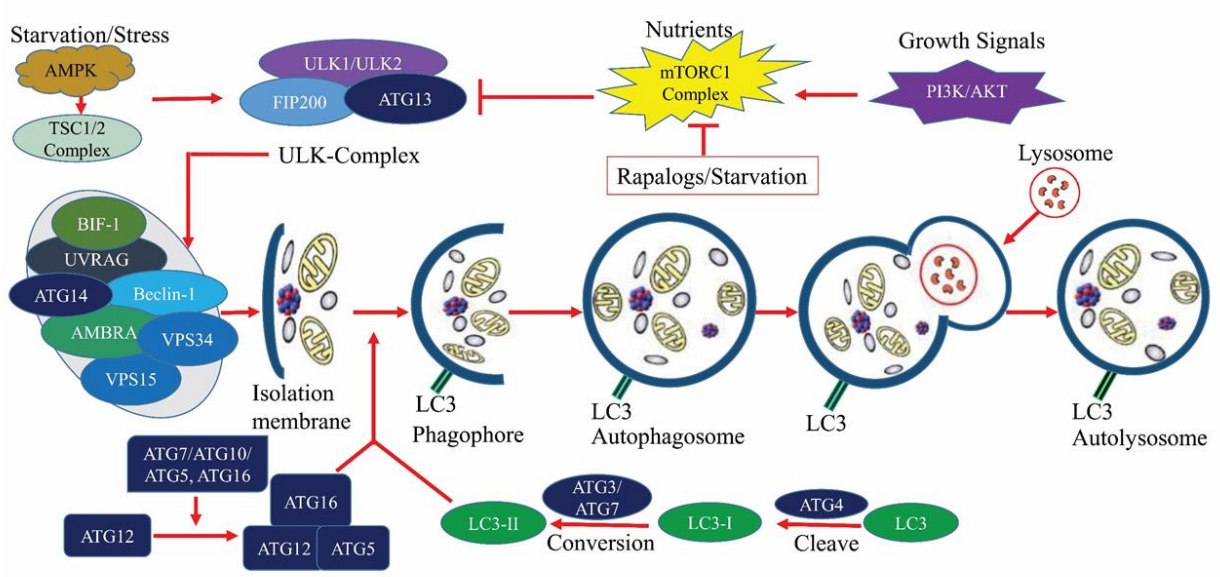


Figure 1.4. The molecular signaling of the autophagy process. Autophagy is a critical process of cells, it can be simplified into several steps including, Formation of isolation membrane; Nucleation of phagophore; Maturation into autophagosome and Autolysosome. Under starvation/stress, ATP/AMP activates AMPK which further activates TSC1/2 complex. This complex induces autophagy through the blocking of mTOR. Elongation is controlled by ATG7 and ATG10 mediated covalent conjugation with ATG12 and ATG5. ATG5/ATG12 non-covalently bind with ATG16 to form an ATG12/ATG5/ATG16 complex which subsequently adds LC3 to phagophoric barrier. Circular autophagosome formation is carried out by recycling of LC3 (conversion of LC3I to LC3II by ATG4). Mature autophagosome gradually fuses with lysosome for selective degradation and recycling of nutrients. This figure was based on published data (Kim and Lee, 2014, Cicchini et al., 2015, Ktistakis and Tooze, 2016). It is adopted from our review paper (Islam Khan et al., 2019).

1.5.2.2 Autophagy in cellular homeostasis

The degradative mechanisms of autophagy maintain homeostasis by digesting intracellular proteins, folded components, and various organelles. The damaged proteins and organelles gradually accumulates in cells or tissues. However, the excessive accumulation can causes toxic effects to cells where basal autophagy plays an important roles in digesting them and maintaining intracellular balance and cellular processes (Mizushima and Komatsu, 2011). To understand the biological process of autophagy such as degradation and recycling, it is important to understand the substrate and proteomes associated with the processes (White, 2015). For instance, cells from liver, brain, muscle are highly rely on autophagy process to maintain homeostasis by preventing accumulating of damaged cells, proteins and organelles (Mizushima and Komatsu, 2011). The accumulation of cellular by products can causes in autophagy dysregulation. Mammalian cells and yeast cells promote autophagy during starvation or cellular stress condition for their surviving and recycling homeostasis regulating metabolic pathways (Rabinowitz and White, 2010). Therefore, nutrient deprivation or stress can dysregulate the metabolic pathways and induce autophagy to restore homeostasis in cells. The cells or tissue specific autophagy is very complex in normal cellular condition where it can promote various pathogenesis such as central nervous system diseases, hepatocellular diseases, aging, and cancer (Levine and Kroemer, 2008). In cancer, a considerable number of findings revealed that autophagy play dual roles in cancer by either promoting or inhibiting cancer cell proliferation, migration, and progression (White, 2012).

1.5.2.3 Autophagy in tumour promotion

In cancer, the cells undergoes nutrient deprivation and stress especially when the tumour is expanding quickly. The excessive growth and proliferation demands higher metabolic and biosynthetic needs (Degenhardt et al., 2006). For instance, the hypoxic tumours microenvironment

promote autophagy to maintain tumour cell survival. In addition, RAS-transformed cancer cells induced autophagy to enhance their growth, proliferation, invasion, and metastasis (Guo et al., 2011, Lock et al., 2011, Yang et al., 2011, Lock et al., 2014). Zheng and co-workers revealed that autophagy induction can promote aggressiveness of CRC cells by adopting apoptotic stimulus (Zheng et al., 2012). Sato K and colleagues revealed that autophagy induction in CRC cells promotes tolerance of nutrient stress resulting in cell survival and transforming metastasis (Sato et al., 2007). It is well established that metastatic CRC eventually develop resistance against chemotherapy. The tumours genes such as EGFR, RAS, BRAF, and p53 are key signalings in the development of chemoresistance (Koustas et al., 2018). PI3K/AKT/mTOR are key elements of EGFR signaling which regulate autophagy in most cancers (Tan et al., 2016). Many studies reported that BRAF mutation induce autophagy in CRC through upregulating LC3B and Beclin-1(Koustas et al., 2017). Apart from this, anti-EGFR monoclonal antibodies such as, cetuximab and panitumumab can also induce autophagy in CRC cells (Koustas et al., 2017).

1.5.2.4 Autophagy in tumour suppression

Autophagy has also been proposed in the process of its tumour suppressor mechanism (White, 2015). The concept derived from early research findings where ATG6 and BECN1 were degraded about 50% in PRC, BRC, and ovarian cancer (OVC) (Aita et al., 1999, Liang et al., 1999, Choi et al., 2013). Indeed, considerable research revealed that inhibition of autophagy can enhances tumorigenesis where subjects with BECN1 mutation is prone to the formation of liver cancer (LVC), lung cancer (LUC), and lymphomas (Qu et al., 2003, Yue et al., 2003). Equally, autophagy deficiency trigger oxidative stress which promote epigenetic modification resulting in initiation and progression of cancer (Karantza-Wadsworth et al., 2007, Mathew et al., 2007, Mathew et al., 2009). In addition, oxidative stress can activate enzymatic or non-enzymatic antioxidant defences,

and other cellular factors which promote carcinogenesis in most cases (Strohecker et al., 2013). In the hypothesis of tumour suppressive autophagy, genes such as, mTORC1, PI3K, Akt, ERK, and Bcl-2 inhibit autophagy process, whereas tumour suppressor genes such as, PTEN, p53, Beclin-1, UVRAG, and DRAM induce autophagy in tumours (Kung et al., 2011). In addition, many research support previous concept where key autophagy genes including, Beclin-1, UVRAG, and Bif-1 were found to be mutated in various cancer (Yue et al., 2003, Liang et al., 2006, Takahashi et al., 2007). Another study revealed that mutation of various ATGs (ATG2B, ATG5, ATG9B, and ATG12) promote microsatellite instability in gastric cancer (GC) and CRC (Kang et al., 2009). In CRC, autophagy responds differentially where it either promote or suppress autophagy (Lauzier et al., 2019). For example, nutrient deprivation hyperproliferate autophagy deficient CRC cells SW480 by activating mTOR signaling (Lauzier et al., 2019).

1.5.2.5 Autophagy-inducing lncRNAs in cancer

The biological and physiological roles of autophagy-modulating lncRNAs in carcinogenesis are being unveiled recently. The expression of lncRNAs greatly impacts on the extent of autophagy at different carcinogenic stages, mostly in advanced metastatic stages. A number of research articles suggested that lncRNAs induce or suppress autophagy through ATGs and their signaling pathways. The complex process of autophagy modulation by expressions of lncRNAs may suppress or promote carcinogenesis under diverse physiological conditions (Figure 1.5). Here we described below the recently characterized lncRNAs and their mechanisms through inducing or inhibiting autophagy in different cancers. These genes have also been summarized in our previous review article (Table 1.1) (Islam Khan et al., 2019).

Majority of the autophagy-modulating lncRNAs have a positive relationship with the induction of autophagy. Hence, increase expression of these lncRNA in tumour induced autophagy and decrease expression of these lncRNA suppressed autophagy. Some examples are listed as follow:

HOTAIR: HOTAIR contains 2158 nucleotides and was discovered by Rinn and co-workers. It is located on intergenic space of HOXC11 and HOXC12 in chromosome 12q13.13 (Rinn et al., 2007). Abnormal expression of HOTAIR has been found for most cancers including, brain tumor (BT), BRC, CRC, GC, LVC, NSCLC, OVC, and pancreatic cancer (PNC) (Loewen et al., 2014, Zhou et al., 2014, Miao et al., 2016, Gerardo et al., 2017). Several research groups have reported HOTAIR association in different cancer evolutionary processes including, epithelial-mesenchymal transition (EMT), TNM staging, prognosis, drug resistance, metastasis, DFS, OS, and tumor development (Loewen et al., 2014, Zhou et al., 2014). Recently, a growing number of studies have revealed HOTAIR contexts in the regulation of autophagy, cancer progression and drug resistance (Yang et al., 2016, Bao et al., 2017, Sun et al., 2017). Liu's research group proved that, upregulated HOTAIR in HCC cells and tissues induce autophagy by promoting two major ATG3 and ATG7 (Yang et al., 2016). It is also proved that, HOTAIR expression increases along with signal transducer and activator of transcription 3 (STAT3) and ATG12 (key of autophagosome formation) through suppressing the cancer suppressing micro RNA miR-454-3p in chondrosarcoma (Bao et al., 2017). Sun and co-workers revealed that HOTAIR abundancy in endometrial cancer (ENDC) cells significantly induce autophagy which controls the development of chemo-resistance towards cisplatin through the expression of Beclin-1 and P-glycoprotein (Sun et al., 2017).

MALAT1: Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is located on chromosome 11q13, containing over 8.7 kb nucleotides and was first identified in NSCLC since 2003 (Ji et al., 2003b). It has been proven that MALAT1 plays significant roles in the development,

proliferation, invasion and metastasis of bladder cancer (BDC), BRC, CRC, HCC, LUC, NSCLC and osteosarcoma (OSTS) (Ji et al., 2003b, Gutschner et al., 2013, Hou et al., 2017, Lupattelli et al., 2017, Zuo et al., 2017, Xiong et al., 2018). Upregulated MALAT1 promote proliferation and metastasis of PRC cells and tissues (Li et al., 2016b) via inducing autophagy. To facilitate the process, MALAT1 interacts with RNA binding protein HuR to activate autophagy via controlling post-transcriptional effects of cytotoxic granule-associated RNA binding protein TIA1 (Li et al., 2016b). It has also been postulated that, aberrant expression of MALAT1 modulates autophagy in various cancers including glioma, GC, HCC, and retinoblastoma by controlling micro RNAs miR-216b, miR-101, miR-124, and miR-23b-3p (Yuan et al., 2016, Fu et al., 2017, Huang et al., 2017a, YiRen et al., 2017). To maintain homeostasis of the cancer microenvironment, upregulated MALAT1 induces conserved autophagy process directly or indirectly to take part in the progression of chemo-resistance and multi-drug resistance (Yuan et al., 2016, YiRen et al., 2017). More recently, Yiren and colleagues revealed that MALAT1 regulates GC progression and autophagy-mediated chemo-resistance via controlling micro RNA miR-23b-3p (YiRen et al., 2017). Gao and colleagues demonstrated that MALAT1 is highly expressed in multiple myeloma (MM) along with high-mobility group box 1 protein (HMGB1) to promote carcinogenesis by significantly expressing two key autophagy regulatory proteins LC3B and Beclin 1 (The mammalian orthologue of yeast ATG6) (Gao et al., 2017a). Their *in vivo* investigation suggests that knockdown of MALAT1 would effectively inhibit MM growth by autophagy suppression (Gao et al., 2017a).

PVT1: Plasmacytoma variant translocation 1 (PVT1) was first identified in murine leukemia virus-mediated T lymphomas. It contains 1716 nucleotides and is located on chromosome 8q24.21 (Zeidler et al., 1994). After its discovery, the roles of PVT1 have been identified in various cancers,

including BDC, BRC, LUC, malignant pleural mesothelioma (MPM) and NSCLC (Cui et al., 2016, Guo et al., 2017, Li et al., 2017b, Lu et al., 2017, Zhou et al., 2017, Guo et al., 2018, Li et al., 2018b). Ma and coworkers proved that PVT1 is significantly upregulated in glioma microvascular endothelial cancer (GMEC) and promote Atg7 and Beclin-1 expression. They reported that excessive endothelial cell proliferation and migration is mediated by PTV1/Atg7/Beclin-1 (Hocker et al., 2017). Huang and co-workers revealed that PVT1 directly activates ULK1, an autophagy activating protein, in pancreatic ductal adenocarcinoma (PDA) cells, patients sample, and *in vivo* xenograft model. PVT1 promotes pathogenesis by regulating miR-20a-5p (Huang et al., 2018a). Thus, PVT1/ULK1/autophagy/miR-20a-5p may be a novel therapeutic target of PDA.

H19: Maternally expressed non-protein coding transcript H19 is lying on the imprinted region of chromosome 11p15.5 and it is 2.3 kb in length (Cui et al., 2002). H19 is transcribed by RNA polymerase II and dysregulation of H19 is associated with BRC, CRC, GC, glioblastoma, HCC, head and neck cancer (HNC), LUC and NSCLC (Matouk et al., 2016, Miyo et al., 2016, Chen et al., 2017c, Luo et al., 2017). In 1990, Brannan and co-workers first discovered H19 as a riboregulator (Brannan et al., 1990). To date, numerous evidence has been established for H19's association in various human cancers through distinctive molecular pathways (Chen et al., 2016a, Chen et al., 2017c). Expression of H19 was found to be increased in both papillary thyroid carcinoma (PPTC) cells and tissues along with estrogen receptor β which may trigger autophagy through regulating ROS and ERK1/2 pathways. Higher expression of H19 promotes PPTC pathogenesis where further investigations may lead to better understanding of PPTC carcinogenesis through H19/autophagy regulation (Li et al., 2018a).

Others: Increasing number of autophagy-modulating lncRNAs are being identified but some of them attracted less attention. Wang and colleagues described, BRAF-activated long non-coding

RNA (BANCR) activate autophagy and contribute to proliferation and apoptosis of both PPTC cells and tissues (Wang et al., 2014b). The overexpression of BANCR promotes conversion of LC3-II/LC3-I, which activated autophagy, promotes cell growth and reduces apoptosis in G1 phase (Wang et al., 2014b). LncRNA-p21 is a hypoxia-responsive intergenic non-coding RNA which is highly expressed in hepatoma and glioma (Işın et al., 2015, Shen et al., 2017). Overexpression of lncRNA-p21 is associated with autophagy induction in hepatoma and glioma cells through HIF-1/Akt/mTOR/P70S6K pathways, resulting in excessive proliferation, motility, reduced apoptosis, and reduced radiosensitivity. Therefore, knockdown of lncRNA-p21 is a new target of radiotherapy as its knockdown potentially alters the molecular events and increases radiosensitivity of hypoxic tumor cells (Shen et al., 2017). A natural antisense transcript of HNF 1A (HNF 1A-AS1) is located on chromosome 12. It is associated with larger tumor size, advanced TNM stage, excessive growth and apoptosis process of HCC cells and tissues through sponging tumor suppressor miR-30b-5p and inducing autophagy (Liu et al., 2016b). Yu et al. recently noticed lung cancer progression associated transcript 1 (LCPAT1) lncRNA in LUC which is directly regulated with autophagic flux (Yu et al., 2018). The overexpression of LCPAT1 and LC3 were found to be in both LUC cells and tumor samples which accelerate the autophagic flux formation to promote carcinogenesis. Whereas, knockdown of LCPAT1 can significantly reduce *in vivo* tumor size by reducing LC3, ATG3, ATG5, ATG7, ATG12, ATG14, and Beclin1 expression (Yu et al., 2018). Chen and co-workers shown that pseudogene of tumor suppressor gene PTEN (PTENP1) is a lncRNA which is downregulated in HCC and sleeping beauty based hybrid baculovirus vectors mediated insertion of PTENP1 could potentially work as targeted anti-tumor agent in HCC cells by reducing proliferation and migration by activating autophagy in PI3K/AKT pathways (Chen et al., 2015).

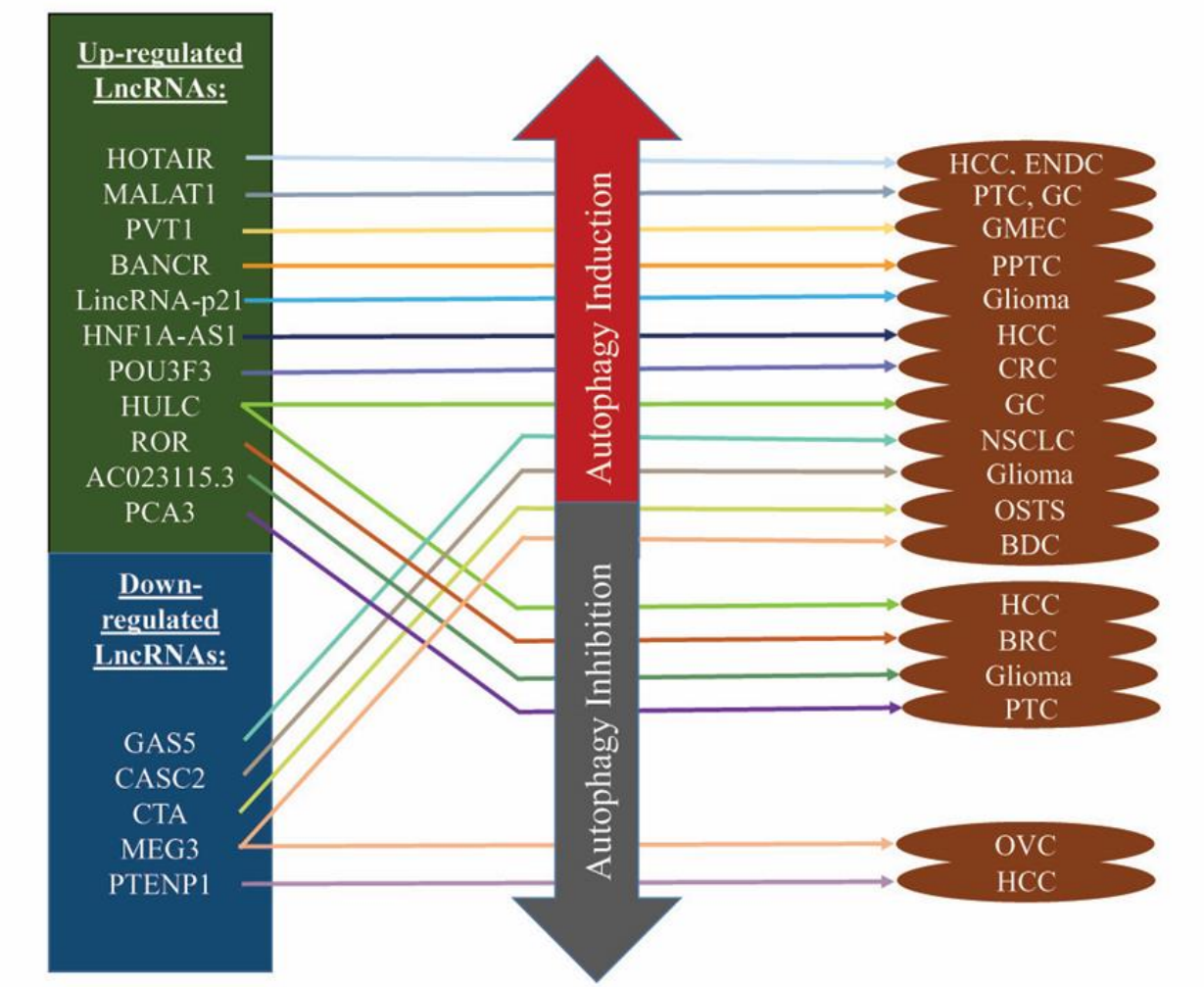


Figure 1.5. Autophagy-modulating lncRNAs in Cancer. Most of the lncRNAs are overexpressed and they induce autophagy to promote/inhibit carcinogenesis. Some of them play key roles in drug resistance while some inhibit autophagy to maintain homeostasis in cancer micro-environments. In the contrary, downregulated lncRNAs induce/suppress autophagy to promote/inhibit cancer progression.

1.5.2.6 Autophagy-inhibiting lncRNAs in cancer

MEG3: Some lncRNA has an inverse relationship with autophagy and one example that has been studied extensively is maternally expressed gene 3 (MEG3). MEG3 is an imprinted gene which was first identified in 2000 (Miyoshi et al., 2000). It contains ~1600 nucleotides and is found in 14q32.3 chromosomal position (Ma et al., 2018). Extensive research demonstrated that MEG3 expression is significantly reduced in cancer and it affects the proliferation, migration and metastasis of most cancers including BRC, CRC, GC, glioma, HCC, LUC, NSCLC and PNC (Gong and Huang, 2017, He et al., 2017, Wang et al., 2017a, Wei and Wang, 2017, Zhang et al., 2017, Ma et al., 2018). Ying et al. speculated that MEG3 inversely regulates cellular autophagy process via the p53 pathway, and reduced MEG3 induces autophagy to promote BDC proliferation and progression (Ying et al., 2013). Downregulation of MEG3 promote tumorigenesis and progression of epithelial OVC cells proliferation and colony formation through inhibiting autophagy process (Xiu et al., 2017). On the other hand, upregulation of MEG3 inhibits the expression of autophagy-related proteins LC3, ATG3, and LAMP1(Xiu et al., 2017). These findings led to the development of MEG3 as a potential biomarker of early diagnosis and treatment of OVC. More recently, Ma et al. also proved that MEG3 is associated with cisplatin-induced glioma cells death by regulating autophagy (Ma et al., 2017a).

Others: Jiang and co-workers explored a novel relationship of cancer susceptibility candidate gene 2 (CASC2) in temozolomide (chemotherapy drug) resistance of glioma (Jiang et al., 2018). CASC2 is negatively downregulated with miR-193a-5p in temozolomide resistant glioma tissues and induce autophagy by controlling mTOR expression to promote drug resistance (Jiang et al., 2018). Ma and colleagues have described lncRNA AC023115.3 upregulation in glioma cells after cisplatin treatment and induce cisplatin-mediated apoptosis by inhibiting autophagy process via

miR-26a/GSK3β axis (Ma et al., 2017b). Wang and colleagues showed that lncRNA CTA is significantly downregulated in OSTS cells and cancer tissues in contrast with the adjacent normal tissues. Downregulated CTA expression is also associated with the advanced TNM stage, larger tumor size and reduced chemosensitivity of doxorubicin through autophagy process (Wang et al., 2017e). Micro RNA miR-210 is negatively regulated with CTA in OSTS and promote apoptosis of OSTS cells, whilst overexpression of CTA inhibits autophagy and subsequently sensitizes to doxorubicin in both *in vitro* and *in vivo* (Wang et al., 2017e). Prostate cancer antigen 3 (PCA3) is a newly identified lncRNA, located on chromosome 9q21-22 and highly specific for PRC (Popa et al., 2007). He et al. reported that PCA3 is overexpressed in PRC to promote proliferation, migration, and invasion by sponging miR-1261 through inhibiting protein kinase D3 (PRKD3) and blocking protective autophagy (He et al., 2016). On the other hand, silencing of PCA3 is able to induce protective autophagy and lessen the PTC progression which could be a novel target of personalized treatment (He et al., 2016). Shan's team established that, silencing of lncRNA POU3F3 could be a novel therapeutic target of CRC as itsignificantly reduce CRC cells proliferation, migration and activate autophagy process by enhancing the expression of autophagy-related genes and proteins Beclin-1, ATG5, ATG7 and LC3 II (Shan et al., 2016).

1.5.2.7 Autophagy-modulating lncRNAs either induce or inhibit autophagy in cancer

In the literature, we found some reports describing the same lncRNA but with opposite relationship with autophagy. Here we include three examples which have been studied extensively.

HULC: Highly upregulated in liver cancer (HULC) was an lncRNA originally characterized in HCC as a significantly overexpressed lncRNA (Panzitt et al., 2007). HULC contains two exons, 1.6k nucleotides in length, located on chromosome 6p24.3. It is significantly dysregulated in most of the cancers including CRC, GC, HCC, OSTS and PNC (Panzitt et al., 2007, Chen et al., 2017d,

Li et al., 2017d, Shaker et al., 2017, Tarrado-Castellarnau et al., 2017, Yu et al., 2017b). A number of groups addressed HULC dysregulation and its molecular mechanisms in various cancers including, proliferation, migration, apoptosis, and metastasis but limited reports have focused on autophagy. Zhao and co-workers established that overexpression of HULC is clinically correlated with the developmental process of GC by promoting proliferation, migration, invasion and reducing cellular apoptosis from autophagy induction (Zhao et al., 2014). Xiong and colleagues found that HULC overexpression induces autophagy and resulting in reduced chemosensitivity of the potent chemo drugs 5-fluorouracil, oxaliplatin and pirarubicin in HCC cells (Xiong et al., 2017). Moreover, inhibition of protective autophagy by silencing of HULC sensitizes these three drugs activity through controlling silent information regulator 1 (sirt1) protein in HCC (Xiong et al., 2017). In the contrary, Chen et al. reported that HULC suppresses *in vitro* apoptosis and *in vivo* tumor development through autophagy blockage in epithelial ovarian carcinoma (Chen et al., 2017b). Upregulated HULC inhibits expression of ATG7, LC3-II, and LAMP1 while activates SQSTM1/p62 to promote carcinogenic events (Chen et al., 2017b).

GAS5: GAS5 was first identified in 1988. It is a tumor suppressor lncRNA which contains 630 nucleotides and encoded at chromosome 1q25 (Schneider et al., 1988). So far, it is well established that GAS5 plays key roles in diverse molecular functions in cancers (Pickard and Williams, 2015, Ma et al., 2016a, Gao et al., 2017b). Meta-analysis of GAS5 shown that, it is associated with DFS, OS, lymphatic node metastasis (LNM) and tumor stages (I, II, III, IV) (Gao et al., 2017b). GAS5 is well known for the negative regulation of most cancer cells the survival (Song et al., 2014, Chen et al., 2018). Zhang and co-workers have demonstrated that downregulating GAS5 would inhibit autophagy in NSCLC and facilitate drug resistance. Overexpression of GAS5 through vector mediated transfection induced autophagy and promoted chemotherapy (cisplatin) response in

NSCLC cells (Zhang et al., 2016b). Gu and colleagues reported that GAS5 expression and autophagy were both downregulated in BRC cells and patients sample. GAS5 expression is negatively correlated with tumor size, advanced TNM and poor prognosis of diseases (Gu et al., 2018). Interestingly, vector-mediated overexpression of GAS5 triggers autophagy and increases LC3, ATG3, and p62 expressions through sponging miR-23a. These findings may be developed into a targeted therapy for BRC through GAS5/miR-23a/ATG3 axis (Gu et al., 2018). However, another experiment conducted by Huo's group also demonstrated reduced GAS5 expression in cisplatin-resistant glioma cell lines. Further investigations on the mechanisms have shown that GAS5 downregulated glioma cells become resistant to cisplatin by increasing autophagosomes formation (Huo and Chen, 2018).

ROR: The lncRNA regulator of reprogramming (ROR) was first identified in pluripotent stem cells. It contains 4 exons, totally 2.6 kb in length and is located on chromosome 18q21.31 (Loewer et al., 2010, Zhan et al., 2016). ROR has been reported to be involved in isolated cellular processes, including growth, proliferation, migration, apoptosis, autophagy and metastasis of BRC, CRC, GC, HCC, nasopharyngeal carcinoma (NPC) and PNC cancers (Takahashi et al., 2014, Pan et al., 2016, Wang et al., 2016b, Zhan et al., 2016, Li et al., 2017a, Peng et al., 2017, Wang et al., 2017d). Chen & co-workers (Chen et al., 2016b) proved that ROR suppresses autophagy and gemcitabine-induced cell death in BRC cells by regulating miR-34a. In another study, however, Li's team showed that ROR is upregulated in PNC to promote basal autophagy which suppresses pyruvate kinase isozymes 2 (PKM2) and reduce chemo-sensitivity (gemcitabine) of cells (Li et al., 2016a). Li and colleagues also identified ROR overexpression reduce autophagy to increase proliferation, invasion, migration and tamoxifen resistance in BRC cells and tissues (Li et al., 2017c). On the

other hand, silencing of ROR effectively increases the sensitivity of tamoxifen, decreases proliferation and migration by inducing autophagy (Li et al., 2017c).

Table 1.1. List of autophagy-modulating lncRNAs and their roles in various cancer

LncRNAs	Expression in cancer	Autophagy modulation	Functions	References
HOTAIR	Upregulated	Induce autophagy	Cancer progression and drug resistance in HCC and ENDC.	(Yang et al., 2016, Bao et al., 2017, Sun et al., 2017)
MALAT1	Upregulated	Induce autophagy	Promote cell proliferation, metastasis and autophagy induced drug resistance in MM, PTC and GC.	(Li et al., 2016b, Yuan et al., 2016, Fu et al., 2017, Gao et al., 2017a, Huang et al., 2017a, YiRen et al., 2017)
MEG3	Downregulated	Induce autophagy/ Inhibit autophagy	Promote BDC proliferation, colony formation and progression through inducing autophagy; while OVC proliferation and progression is raised inhibiting autophagy.	(Ying et al., 2013, Xiu et al., 2017)
HULC	Upregulated	Induce autophagy/ Inhibit autophagy	Promote GC proliferation, migration, and invasion and reduced apoptosis by inducing autophagy; chemosensitivity of HCC is promoted by inhibiting autophagy.	(Zhao et al., 2014, Xiong et al., 2017)
GAS5	Downregulated	Induce autophagy	Progression of NSCLC and cisplatin resistance.	(Zhang et al., 2016b)
ROR	Upregulated	Inhibit autophagy	Promote proliferation, invasion, migration, tamoxifen resistance and reduced gemcitabine induced cell death in BRC.	(Chen et al., 2016b, Li et al., 2017c)

Table 1.1. List of autophagy-modulating lncRNAs and their roles in various cancer (continued)

LncRNAs	Expression in cancer	Autophagy modulation	Functions	References
PVT1	Upregulated	Induce autophagy	Promote GMEC proliferation migration and angiogenesis.	(Hocker et al., 2017)
BANCR	Upregulated	Induce autophagy	Promote PPTC proliferation, growth and reduce cell population at G1 phase.	(Wang et al., 2014b)
CASC2	Downregulated	Induce autophagy	Glioma progression and temozolomide resistance through controlling mTOR.	(Jiang et al., 2018)
LincRNA-p21	Upregulated	Induce autophagy	Promote proliferation, motility, reduce apoptosis and radio-sensitivity in hepatoma and glioma.	(Işın et al., 2015, Shen et al., 2017)
AC023115.3	Upregulated	Inhibit autophagy	Increase cisplatin mediated apoptosis in glioma.	(Ma et al., 2017b)
CTA	Downregulated	Induce autophagy	Promote TNM staging, larger tumor depth and reduced chemosensitivity in OSTS.	(Wang et al., 2017e)
PCA3	Upregulated	Inhibit autophagy	Increase proliferation, migration and invasion in PTC	(He et al., 2016)
HNF1A-AS1	Upregulated	Induce autophagy	Promote TNM staging, increase growth and apoptosis in HCC.	(Liu et al., 2016b)
PTENP1	Downregulated	Inhibit autophagy	Promote proliferation and migration in HCC.	(Chen et al., 2015)
POU3F3	Upregulated	Induce autophagy	Promote CRC proliferation and migration.	(Shan et al., 2016)

1.5.3 Proteomics-based cancer biomarkers

Proteins are dynamic regulatory components of cells to maintain the cellular processes. Under normal condition, proteins are distributed throughout the body in their native structure and maintain homeostasis by regulating cellular complex pathways. The normal functions of proteins or proteostasis may be dysregulated in response to stress resulting in abnormal housekeeping activity to the cells (Hedl et al., 2019).

Among all types of biomarkers, proteins can be detected at nanograms to micrograms levels. This high sensitivity of detection made them as excellent potential candidates of biomarkers for individual type of diseases (Doustjalali et al., 2014, Hedl et al., 2019). According to definition, diagnostic biomarkers can be used to detect pathogenesis in their early stage, whereas prognostic biomarkers allow us to predict disease outcome. The term therapeutic biomarkers, however, refers to the proteins that could be used for treatment (Carlomagno et al., 2017). The appropriate categorization of biomarkers are essential in drug design and delivery to its target site (Hamdan, 2007).

Even through, the theory of abnormal protein expressions associated with various pathogenesis was well accepted, the term proteomic cancer biomarker was only introduced in the last decade after advancement of technologies related to protein study (Li and Chan, 2014, Alharbi, 2020). The area of proteomics mostly studied structure and functions of proteins, functions during pathogenesis and nature of individual proteins in each cell type. The proteomics biomarkers are usually protein components derived from body's own system during normal or pathological conditions and can be efficiently identified and detected using techniques such as mass spectrometry (MS) (Li and Chan, 2014). The approach of proteomics biomarkers can be used in

not only cancer but also a wide variety of diseases such as cardiovascular diseases, immune diseases, diabetes, central nervous system disease, kidney diseases, etc. (Albulescu et al., 2019).

To establish a highly specific and sensitive biomarker, advancement of proteomics tools and their detectability from body fluids such as urine, blood, stools, or biopsy samples are very crucial. The using of body fluids is well accepted to the patients because of the non-invasiveness and cost-effectiveness compared to any other methods. However, in spite of recent proteomics technological developments, challenges in proper sampling or sample preparations need to be overcome especially sample collecting from body fluids which contain complex mixture of proteins (Hongzhan et al., 2007, Schubert et al., 2017). There are several advantages of using blood for biomarker study such as high specificity, easier and cost effective sampling procedure, higher stability during analysis and storage, and can be collected less-invasively (Amiri-Dashatan et al., 2018). On the contrary, using blood plasma or plasma proteins for developing or identifying potential biomarkers have several difficulties such as wide variety of protein containments, low abundancy of targeted proteins, and vast patient variation (Geyer et al., 2019, Ignjatovic et al., 2019). In addition, discovery of proteomics-based biomarkers is less reliable when a single tool is being used. Therefore, to improve the sensitivity and specificity of proteomics-based biomarkers a combination of two dimensional gel electrophoresis (2D-PAGE), Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF), Surface-enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF), and Isobaric tag for relative and absolute quantification (iTRAQ) MS techniques are commonly used to establish error free biomarkers (Amiri-Dashatan et al., 2018, Geyer et al., 2019, Ignjatovic et al., 2019).

As discussed in Section 1.5, cancer biomarkers can be anything secreted biologically from body in regular or irregular manner (such as enzymes, hormones, receptors) or genetic alterations which

significantly take parts in carcinogenesis or work as oncogenes (Alharbi, 2020). The identification of proteomic biomarkers for different cancers may improve the diagnosis, prognosis and treatment (Amiri-Dashatan et al., 2018, Albulescu et al., 2019). So far, thousands of proteins have been reported as potential cancer biomarkers, but only a very negligible number were approved by US-FDA for clinical practice. The most commonly used MS identified biomarkers are OVA1, pre-albumin, apolipoprotein A1, and transferrin. In fact, OVA1 was the first FDA approved diagnostic biomarker for OVC (Zhang et al., 2004). Some circulatory proteins such as CA15-3 for BRC, PSA for PRC, and CA-125 for OVC have already been approved by FDA and commercially available for diagnostic purposes, (Kirwan et al., 2015, Ho et al., 2016, Kailemia et al., 2017). Here, we summarized the proteomics-based cancer biomarkers proposed by recent studies for common cancers according to their global incidence.

1.5.3.1 Proteomics-based biomarkers for LUC

The most prevalent cancer worldwide is LUC. The number of new cases and mortality are increasing every day and most cases are caused by smoking, second-hand or passive smoking, inhalation of radon gas, exposure to chemical or environmental pollution, or genetic factors (Malvezzi et al., 2018). The poor surviving rates of LUC compared to other tumors such as CRC, BRC, is due to the lack of early detection or delayed diagnosis of tumor in advanced stages (Aberle et al., 2011). However, National lung screening and NELSON trial demonstrated that low-dose computed tomography (LD-CT) can reduced LUC mortality for about 20-30%. Therefore, US preventive services suggested LD-CT annually for high-risk populations (Gasparri et al., 2020). Recent advancement of technologies have also been used to explore proteomics-based biomarkers. (Hoseok and Cho, 2015). MS is a very powerful tool using the mass to charge conversion analysis to determine the presence of proteins from a complex mixtures and further compared with global

proteomics data to established biomarkers (Malvezzi et al., 2018, Gasparri et al., 2020). The MS-based biomarkers identified by liquid chromatography (LC) are widely accepted for quantitative data, high reliability and equitability. It can measure the protein existence from tiny samples without the requirement of prior knowledge of the proteins. In addition, it can identify proteins in their various forms, fragments, phosphorylated proteins and post-translationally altered proteins associated with pathogenesis (Singh et al., 2017). The proteomics biomarkers discovery has started since 1990, and the outcomes, accuracy and sensitivity improved dramatically in recent years (Singh et al., 2017, Gasparri et al., 2020). In Table-1.2, we summarized the recent findings of proposed proteomics cancer biomarkers for LUC. Various samples sources such as bronchoalveolar lavage, plasma, serum, and tissue biopsy were being analyzed using different MS tools.

Recently, two studies have been conducted on bronchoalveolar lavage samples from LUC patients to establish diagnostic biomarkers using LC-MS/MS techniques. They concluded that chaperonin containing TCP1 subunits 2-4, CST3, LCN2, and MMP1 may serve as promising diagnostic biomarkers for LUC (Carvalho et al., 2017, Hmmier et al., 2017). Likewise, another study used 2D-PAGE separation and MALDI-TOF/TOF to analyze bronchoalveolar lavage samples and identified AKR1B10, HSP70, PKM2, and PRDX1 as high fold-changed proteins (Pastor et al., 2013). Uribarri et al., proposed APOA1, CO4A, CRP and GSTP1 expression for higher diagnosis accuracy with 95% and 81% specificity and sensitivity which may be used in clinical settings of LUC diagnosis (Uribarri et al., 2014). The recent advancement of proteomics techniques has involved a numbers of new platforms such as iTRAQ, liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI MS/MS), and SELDI-TOF MS/MS that can effectively and precisely separate the proteomes in any biological samples of LUC patients

including plasma, serum, tissue biopsy apart from broncholveolary lavage (Poschmann et al., 2009, Pastor et al., 2013, Giusti et al., 2014, Kim et al., 2015b, Wang et al., 2016a, Hocker et al., 2017).

Table 1.2. Proteomics-based biomarkers for LUC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Bronchoalveolar lavage	LC-MS/MS	CST3, LCN2, MMP1, and TCP1 subunits 2-5	Different stages LC samples	(Carvalho et al., 2017, Hmmier et al., 2017)
Bronchoalveolar lavage	2D-GE + MALDI-MS	AMY1A, AMY2A, ANXA1, ANXA5, APOA1, ARHGDIB, C3A, CAI, CAPS2, CFL1, CO4A, CRP, ENO1, GSR, GSTA1, GSTP, GSTP1, HSP70, IDH1, LCN2, PEBP4, SAMP, SERPINB1, STMN1, TPPP3, TXN, UCHL1	Missing female patients, Second cohort for NSCLC	(Pastor et al., 2013, Uribarri et al., 2014)
Plasma	2D-GE + MALDI-MS	PRX1, PRX2, SCGB3A2, and TPM4	Small number of samples with different stages samples	(Rostila et al., 2012, Li et al., 2018b)
Plasma	iTRAQ+2D-GE LC/MS, LC/MS, MALDI MS	ACTN1, ALDOA, ENO1, FLNA, G6PD, HSP90B1, ICAM1, ILK, LDHB, MSN, PGI, PGK1, PKM2, SCGB3A2, SPP1, TALDO1, THBS1, ZYX, and proteins peak at 11493, 6429, 8245, 5335, 2538 Da	Late stage diagnosis with different stages LC samples	(Shevchenko et al., 2010, Kim et al., 2015b, Li et al., 2018b)
Serum	2D-GE+MALDI MS, LC-ESI MS/MS	CA1 and a group of > 30 peaks	Different stage samples	(Wang et al., 2016a, Hocker et al., 2017, An et al., 2019)
Serum	MALDI-TOF-MS, MALDI-MS	C3 protein, ERF3A, Haptoglobin B-chain	Proper staging missing	(Du et al., 2011, Ayyub et al., 2015)
Tissue biopsy	2D-GE + LC/MS	ALDOA, CCND1, CKS, CyPA, HSP47, MPM, MYL2, MYL3, MYL6B, TAGLN2, TGF B, and VIM	Mixed samples of mesothelioma, lung and bronchial epithelium	(Poschmann et al., 2009, Rho et al., 2009, Giusti et al., 2014)

1.5.3.2 Proteomics-based biomarkers for BRC

BRC is the deadliest cancer for women worldwide which accounted 14.0% of cancer-associated death in female globally (Youlden et al., 2012). The 5-year survival of BRC is poor due to late diagnosis. The survival was remarkably improved by more than 90.0% in western countries if the cancer is detected in earlier stages (Youlden et al., 2012). The aggressiveness of BRC is mostly associated with the expression of various receptors such as human epidermal growth factor receptor 2 ($HER2^+$), estrogen receptor (ER^+), progesterone receptor (PR^+), and the absence of hormone receptor (HR^-) (Feng et al., 2018, American Institute for Cancer Research, 2020). The classification of BRC sub-types is based on the sample types, histopathology, protein contents, and whole genome characteristics of tissues (Anderson et al., 2014). The genetic heterogeneity of the BRC tissues are differentially regulated by individual cellular responses in the tumor microenvironment. Therefore, the difference in biomarkers can vary with the clinical variables, diverse heterogeneity and progression of BRC (Mueller et al., 2018). Mechanistically, extensive roles of proteins in cellular processes, homeostasis, and metabolism are regulated by proteolytic degradation, translational modification and structural formation of proteins (Sallam, 2015). Thus, collecting and proteins profiling of BRC samples from any biological fluids or tissues can promote better understanding of pathogenesis, progression and novel therapeutic targets of the diseases (Sallam, 2015). Similar to other high-throughput sequencing techniques, proteomics generates big datasets of proteins where the abnormal expressions of a particular of group of proteins are found to be responsible for the BRC pathogenesis. The precise analysis of MS data has established a list of novel biomarkers for BRC management (Wilhelm et al., 2014). As summarized in Table 1.3, studies using BRC tissues revealed distinct groups of aberrantly expressed proteins where CA15-3, fibrinogen, fibronectin, SAP, and TSP5 comprised for diagnostic biomarkers of BRC with

improved accuracy (Deng et al., 2006, Zeng et al., 2011, Dowling et al., 2014, Abdullah Al-Dhabi et al., 2016, Yigitbasi et al., 2018). Similarly, a list of diversely expressed proteins has been identified from BRC plasma and serum samples which might be used in clinical settings with further verification (Pitteri et al., 2010, Washam et al., 2013, Zeidan et al., 2015, Pendharkar et al., 2019). Another novel findings of Yang and co-workers revealed that the detection of N-/O- linked galatosylated glycans in saliva can provide distinguished information among benign BRC and Stage-I/II BRC patients. It could be a potential non-invasive early diagnostic biomarkers for BRC (Yang et al., 2020).

Table 1.3. Proteomics-based biomarkers for BRC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Plasma	LC-MS/MS	EGFR, Gentisic acid, Myoinositol, Proline, 2-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid	Post-menopausal women; used metabolic products	(Pitteri et al., 2010, Jasbi et al., 2019)
Plasma	SELDI-TOF MS	13 bone metastasis biomarkers were identified where circulating PTHrP(12-48) reached > 85.0% specificity and sensitivity	Large cohorts of samples	(Washam et al., 2013)
Saliva	MALDI-TOF/TOF-MS	Galactosylated glycans	Mixed stages samples	(Yang et al., 2020)
Serum	2D-GE + MALDI-MS	A1AT, CA15-3, CATD, DAPLE, GELS, HS90B, IQCC	Histopathological characteristics available	(Fan et al., 2012, Pendharkar et al., 2019)
Serum	SELDI-TOF MS	APOA1, APOA2, APOC2, APOC3, Coagulation factor XIIIa, Haemoglobin α-β chains, and proteins peak at 6,648 Da	Post-operative follow-up maintained; dataset with pilot study	(Opstal - van Winden et al., 2012, Song et al., 2017)
Serum	SELDI-TOF MS + MALDI-TOF MS	ANX A3, Apolipoprotein C-1 peptides, and 14 proteins peak at 9427, 3163, 3972, 6630, 6577, 6429, 6813, 6983, 12635, 4283, 7552, 6450, 6629, and 5171 Da	Used well-defined staging samples	(Böhm et al., 2011, Zeidan et al., 2015, Sun et al., 2016)

Table 1.3. Proteomics-based biomarkers for BRC (continued)

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Serum and tissues	LC-MS/MS	A1BG, CA15-3, CDH5, Fibrinogen, Fibronectin, pIgR, PZP, SAP, TSP1, and TSP5	Various stages of cancer samples; including metastatic samples	(Zeng et al., 2011, Fry et al., 2013, Dowling et al., 2014)
Serum and tissues	SELDI-TOF MS	CA15-3, and proteins peak at 3,972, 6,850, 8,115, and 8,949 Da	Different stages of invasive ductal carcinoma	(Yigitbasi et al., 2018)
Tissues	2D-GE + MALDI-MS	Annexin 1, Beta-catenin, BRG1, Cathepsin V, CST6, CUL5, d-HYD, EDDM3B, Enolase-1, GDH, Human GST, Kinase like protein, MUTS2, PLPP3, Vimentin	Only Stage-II tumours	(Abdullah Al-Dhabi et al., 2016)
Tissues	2D-GE + MALDI-MS	Alpha-1-antitrypsin, Cathepsin-D, EF-1-beta, PSMA1, RPS12, SMT31, TCTP	Less patients number with mixed stages samples	(Deng et al., 2006)

1.5.3.3 Proteomics-based biomarkers for CRC

Local tissue samples can provide most appropriate information about tumor microenvironments. Several LC-MS study revealed a group of proteins where Quesada-Calvo et al., proposed and validated KNG1, OLFM4 and Sec24C as diagnostic biomarkers among 561 differentially expressed proteins (Table 1.4) (Quesada-Calvo et al., 2017). Another studies revealed that ACTBL2, Aldose A, Annexin A2, cyclophilin A, and DPEP1 may also serve as diagnostic biomarkers and new therapeutic targets for the management of CRC (Yamamoto et al., 2016, Ghazanfar et al., 2017, Hao et al., 2017). On the other hand, circulating proteins are widely accepted biomarkers source for any pathological conditions including CRC due to higher abundancies (Chantaraamporn et al., 2020). Chantaraamporn and colleagues revealed that plasma content of complement C9 and fibronectin may provide crucial information of CRC development (Chantaraamporn et al., 2020). Furthermore, Peltier et al., revealed that serum content of SERPINA1, SERPINA3, and SERPINC1 are potential biomarkers candidate for CRC early detection. In this study, the sensitivity and specificity for SERPINA1 and SERPINC1 reached 95% (Table 1.4) (Peltier et al., 2016). More recently, Thorsen and colleague performed 2D gel-based MS study on 128 tumours with its adjacent normal biopsy samples. Their results revealed that TPM3 expressions with its validation in patient plasma samples could be a potential diagnostic biomarkers for CRC (Thorsen et al., 2019). Ludvigsen et al., primarily performed 2D-PAGE LC-MS/MS analysis on CRC cancerous cell line HCT-116 and CRC normal cell NCM460 to establish potential diagnostic biomarkers. The MS analysis and WB validation of S100A4, S100A6, RBP, SET, and HSP90B1 proteins from cell lines and patients samples proposed their use in clinical settings for early diagnosis of CRC (Ludvigsen et al., 2020). Another group performed LC-MS/MS based glycomics study and demonstrated that N-glycome landscape of CRC cells exhibit

aggressive metastatic nature in CRC pathogenesis by regulating EGFR regulatory pathway (Sethi et al., 2016). Another recent study revealed that initial CRC development can be distinguished by MS based post-transcriptional modification analysis of CRC plasma samples (Kopylov et al., 2020). The early stages CRC development is highly associated with dysregulation of cytokines and extracellular matrix proteins and reduction of extracellular matrix stability. The study revealed that APOE, APOC1 and APOB potentially impact of DNA repair through mTOR and PI3K pathways and impact on post transcriptional modification. Therefore, these proteins may be used as potential early diagnostic biomarkers in CRC (Kopylov et al., 2020).

Table 1.4. Proteomics-based biomarkers for CRC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Blood/serum	LC/MRM-MS	AREG, MASP1, OPN, PON3, TFR1	Early stages samples were separated	(Bhardwaj et al., 2019)
Serum	LC-MS/MS	EGFR, HPX, ITIH4, LRG1, and SOD3	Mostly advanced stages CRC patients	(Ivancic et al., 2020)
Serum	MALDI-TOF MS	MST1 and STK4	Mixed adenocarcinoma samples	(Yu et al., 2017a)
Serum	2D LC-MS/MS	MRC1 and S100A9	A complex mixtures of esophageal cancer, gastric cancer, and CRC samples used. Absences of proper histological characteristics of patients	(Fan et al., 2016)
Serum	iTRAQ/MALDI-TOF MS	SERPINA 1, SERPINA 3, and SERPINC1	Well characterized staging of samples	(Peltier et al., 2016)
Tissues	2D-GE + MALDI-MS	ACTBL2	Well characterized pathological data available	(Ghazanfar et al., 2017)
Tissues	Nano-spray LC-MS/MS	DPEP1	Well characterized histological data available	(Hao et al., 2017)
Tissues and Plasma	LC-MS/MS	Aldolase A, Annexin A2, A1AG1, Complement component-9, Cyclophilin A, Fibronectin, KNG1, OLFM4, and Sec24C,	Mixed stages of patients samples	(Yamamoto et al., 2016, Quesada-Calvo et al., 2017, Chantaraamporn et al., 2020)
Tissue and Plasma	MALDI-TOF	TPM3	The identified biomarker was validated in patient's tissues and serum separately.	(Thorsen et al., 2019)
Cell lines and tissues	2D PAGE, LC-MS/MS	S100A4, S100A6, RBP, SET, and HSP90B1	Different aspect of validation performed	(Ludvigsen et al., 2020)

Cell lines and Tissues	LC-MS/MS based N-glycomics	N-glycomes	Mixed stages of patients	(Sethi et al., 2016)
Plasma	Q exactive-HF	APOE, APOC1, and APOB	Absences of proper staging	(Kopylov et al., 2020)

1.5.3.4 Proteomics-based biomarkers for PRC

PRC is one of the most frequently diagnosed adult malignancies worldwide, counted ~1.3 million new cases in 2018 and more than 300,000 death annually (Ferlay et al., 2018). Based on the global statistics, PRC is the second most frequently diagnosed cancer and fifth cancer related death in male population (Ferlay et al., 2018). Early stages of PRC can be treated by combined chemoradiation therapy but it is very rare to diagnosed PRC in their early stages (Kudryavtseva et al., 2019). Clinically, PRC is diagnosed with digital rectal examination, medical imaging, and the expression of PSA levels (Tanase et al., 2017). Microarrays-based differential gene expression diagnosis has been introduced over the past decades aiming to identify a novel diagnostic and prognostic biomarkers for PRC. Notwithstanding, they failed to be validated for clinical uses due to their poor sensitivity (Chen et al., 2019b, Zhang et al., 2019b). In facilitating the biomarker discovery, the powerful MS tool provided comprehensive advantages by its qualitative and quantitative analysis ability, proteome detection ability, reliability and higher sensitivity (Tanase et al., 2017). Recently, a group using a new technique called sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH)-based proteomics has identified 6 proteins GOT1, HNRNP2AB1, MAPK1, PAK2, UBE2N, and YWHAB and proposed them as potential biomarkers considering their extensive roles in PRC development and transitions (Singh and Sharma, 2020). Another study conducted by Ravipaty et al., revealed that FLNA, FLNB, and KRT19 are novel serum-based diagnostic biomarkers for PRC. Validation of their findings indicated that combination of PSA and proposed markers can effectively identify PRC in both early and advanced stages (Ravipaty et al., 2017). Likewise, Kawakami and colleagues demonstrated that serum exosomal gamma-glutamyltransferase activity could be effectively

distinguished advanced PRC patients from benign prostate hyperplasia patients (Table 1.5) (Kawakami et al., 2017).

Table 1.5. Proteomics-based biomarkers for PRC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Serum, cell lines	LC-MS/MS, SWAT-LC-MS/MS	FLNA, FLNB, GOT1, HNRNPA2B1, KRT19, MAPK1, PAK2, UBE2N, and YWHAB	Absences of proper staging	(Ravipaty et al., 2017, Singh and Sharma, 2020)
Tissues	SELDI-TOF MS	GDF15, PCa-24, and TIMP1	Mixed stages samples	(Zheng et al., 2003, Cheung et al., 2004, LIU et al., 2005)
Tissues, Serum, urine, cell lines	iTRAQ + 2D-GE LS/MS, SID-SRM-MS	AMACR, CRISP3, GGT1, MMP9, PF4V1, PSA, and PSMA	Tissue results are verified in urine and serum	(Garbis et al., 2008, Zhang et al., 2016a, Kawakami et al., 2017, Shi et al., 2017)
Tissues, urine, cell lines	MALDI-TOF MS, 2D-DIGE MALDI-TOF MS	ALDH1A3, ARG2, B/MRP-14, DDAH1, eIF4A3, Ezrin, e-FABP5, HSP60, LMNA, MCCC2, PPA2, Prdx3, Prdx4, SLP2, and SM22	Different stages samples, identify diagnostic markers for metastatic	(Rehman et al., 2004, Pang et al., 2010, Skvortsov et al., 2011, Ummanni et al., 2011, Casanova-Salas et al., 2015)

1.5.3.5 Proteomics-based biomarkers for GC

GC is one of the third leading cancer deaths worldwide, counted ~0.8 million death in 2018 (Bray et al., 2018, Bray et al., 2020). The incidence rate of GC has slightly reduced over the last decade but the 5-year survival became worse with less than 10% in advanced stages of GC. Apart from this, early diagnosis of GC increase the 5-year survival to 50% in developed countries (Luo and Li, 2019). Traditionally, carbohydrate antigen (CA) series CA19-9, CA24-2, CA50, CA72-4, and CA125, and CEA are the most frequently using tumor markers but none of them are being used clinically due to poor specificity and sensitivity (Necula et al., 2019). Moreover, complex heterogeneous nature, genetic mutation, translation, and post-translation alterations of GC are key factors affecting poor prognosis and reduced treatment outcomes (Necula et al., 2019). With the use of advanced technologies in molecular biology, GC is subdivided into four subclasses to improve the early diagnosis and prognosis of the diseases (Cancer Genome Atlas Research, 2014). In addition, the new classification of GC opened a new door for trying novel molecular therapeutic regiments combined with or without immune suppressor to exalt outcomes though early diagnosis (Chivu-Economescu et al., 2018, Necula et al., 2019). The proteomics approach is exceptionally promising as it deals with the functional genomic contents which participate in translational processes of initiation, progression and metastasis of GC (Mohri et al., 2016). Another recent study reported that exosomal tripartite motif-containing protein 3 (TRIM3) is downregulated in GC tumours compared to its adjacent normal samples. The overexpression of TRIM3 can suppress GC cell growth and development of metastasis both *in vitro* and *in vivo* (Fu et al., 2018). Their findings suggested that TRIM3 could serve as potential diagnostic biomarkers for GC as well as efficient delivery of TRIM3 can provide new targets for GC (Table-1.6). A recent research revealed a potential candidate DEK proteins as novel diagnostic and prognostic biomarkers for GC (Lee et

al., 2019). The plasma content of DEK sensitivity is superior to the traditional biomarkers CEA, CA19.9 which suggests DEK could be used in clinical settings. Recently in 2020, Zhou and co-workers identified 11 differentially expressed proteins by applying LC-MS/MS together with tandem mass tags which can effectively isolate GC healthy controls from early stages GC samples (Table 1.6) (Zhou et al., 2020).

Table 1.6. Proteomics-based biomarkers for GC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Gastric juice, Plasma, Serum	LC-MS/MS	ANK1, FOLR2, Gastric juice free amino acid, GRN, LILRA2, MGP, NBL1, OAF, PCSK9, PSTPIP2, RPS27A, SHBG, SOD1, and TRIM3	Inappropriate staging in sample groups	(Loei et al., 2012, Cheng et al., 2018, Fu et al., 2018, Liu et al., 2018, Zhou et al., 2020)
Gastric fluids, Serum, tissues, cell lines	MALDI-TOF MS, 2D-DIGE MALDI-TOF MS, SELDI-TOF MS	AAT, CIP2A, GIF, LPCAT1, PIK3CB, S100A9, and Peaks at Da 2863, 2953, 1945, 2082, 5910, 2873, 3163, 4526, 5762, 6121, and 7778 Da	Well characterized samples	(Li et al., 2012, Wu et al., 2012, Uehara et al., 2016, Wu et al., 2016, Saralamma et al., 2020, Zhu et al., 2020b)
Tissue and plasma, cells lines, mouse plasma	iTRAQ, 2D-GE LS/MS, LC-ESI-MS/MS	ANXA1, DEK, FABP1, FASN, Fibulin-5, GGCT, GLS1, HDAC1, ITIH3, MTA2, NNMT, and UQCRC1	Well characterized pathological staging	(Chong et al., 2010, Wang et al., 2016c, Jiang et al., 2017a, Jiang et al., 2017b, Jiang et al., 2019, Lee et al., 2019)

1.5.3.6 Proteomics-based biomarkers for HCC

In the study of HCC, Guo et al. identified 93 differentially expressed proteins from a patient's cohort using iTRAQ, CD14 expression was validated with a specificity and sensitivity more than 80% to establish an early diagnostic biomarkers for HCC (Guo et al., 2017). Kim and colleagues revealed that fucosylated peptide AFP can be a serum based diagnostic biomarkers for patients with HCC (Kim et al., 2018a). In addition, Ding and co-workers proposed salivary based non-invasive biomarkers for HCC (Ding et al., 2019). They identified 133 differentially expressed biomarkers from HCC patients and adjacent healthy controls. Finally, they sieved and verified SOD2 expressions by ELISA from dataset and concluded that it can be a potential marker for HCC early detection. Likewise, another study demonstrated that AFP and ORM1 abundancy in urine samples can be a potential candidate for non-invasive diagnostic biomarkers for HCC (Zhan et al., 2020b). They ensured a higher predictive value and sensitivity in satisfactory levels about 80%. Alternatively, recent trend of MS identified a number of peptides from serum samples and proposed them for diagnostic biomarkers for HCC in clinical settings. For example, Heo et al. achieved specificity and sensitivity about 80% for early diagnostic HCC marker EIF3A peptides from serum samples, ELISA and WB validation confirmed the use of selected peptides clinically (Heo et al., 2019). Similar to the previous study performed by Zhan's group (Zhan et al., 2020b), Lee's group also validated the presence of AFP in a large number of HCC patients serum samples and verified AFP expression by using RT-qPCR and WBtting techniques (Lee et al., 2020). Furthermore, another study revealed that AFP along with heptoglobin specific N-glycopeptides may achieve greater sensitivity of diagnostic biomarkers > 80% for nonalcoholic steatohepatitis HCC patients (Zhu et al., 2020a). Wu and colleagues revealed that MS-based identification of

miR-224 in HCC serum samples may also be potential diagnostic and prognostic markers in clinical practice (Table 1.7) (Wu et al., 2020)

Table 1.7. Proteomics-based biomarkers for HCC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Serum	Q-TOF, TQMS	AFP and miR-224	Well characterized patients details	(Kim et al., 2018a, Wu et al., 2020)
Serum, Saliva, Urine	iTRAQ	AFP, CD14, ORM1, and SOD2	Limited stages mixed sample	(Guo et al., 2017, Ding et al., 2019, Zhan et al., 2020b)
Cells and serum	LC/ESI-MS/MS	AFP and EIF3A	Absence of full staging details	(Heo et al., 2019, Lee et al., 2020)
Serum	EThcD-MS/MS	N-glycopeptides, i. e., N184_A3G3F1S3, N241_A2G2F1S2, N241_A3G3F1S3, N241_A4G4F1S3, and N241_A4G4F1S4	Well characterized staging	(Zhu et al., 2020a)

1.5.3.7 Proteomics-based biomarkers for esophageal squamous cell carcinoma (ESCC)

Despite of recent advancement in esophageal squamous cell carcinoma (ESCC) diagnosis and treatment, early diagnosis still a key regulator for the effective management of ESCC (Fan et al., 2013). To address the necessity, a study revealed that serological content of three differentially expressed proteins FLNA, TUBB, and UQCRC1 may serve as novel post-genomics putative biomarkers for ESCC early detection (Fan et al., 2013). Shah et al. identified 8 glycoproteins from ESAD patients serum samples with 94% area under curve value and 2 candidates were verified to develop early diagnostic biomarkers using lectin magnetic bead array-based immunoblot from large scale patient cohort (Shah et al., 2015). The application of high-throughput proteomics in ESCC serum samples and subsequent analysis by genetic algorithm model identified AHSG, FGA, and TSP1 circulating peptides for early detection of ESCC with sensitivity and specificity more than 95% from 477 patients (Jia et al., 2016). The screening of patient's plasma sample can also be used to establish an early detection biomarker in ESCC. Zhao and colleagues used ESCC plasma sample and performed a combined method DIGE with MALDI-TOF/TOF which identified AHSG and LRG circulating peptides to provide a framework for early ESCC screening (Zhao et al., 2015). In addition, the combination of iTRAQ and 2D-LC-MS MS identified ECM1 and LUM as potential plasma biomarkers for ESCC patients, followed by WBt to validate the findings *in vitro* (Wang et al., 2017c). Subsequently, another study identified a significant decrease of PA28β from ESCC tissues, the knock-in of PA28β found to be reduced tumor growth and proliferation *in vitro* which might act as a diagnostic and prognostic biomarker for ESCC (Table 1.8) (Chen et al., 2017a).

Table 1.8. Proteomics-based biomarkers for ESCC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Serum	MALDI-TOF	FLNA, TSP1, TUBB, and UQCRC1	Well characterized histological data	(Fan et al., 2013, Jia et al., 2016)
Plasma, cell lines and tissues	MALDI-TOF	AHSG, LRG, PA28β	Limited but characterized pathologically	(Zhao et al., 2015, Chen et al., 2017a)
Serum	Q-TOF	26 lectin–protein candidates	Absence of staging	(Shah et al., 2015)
Plasma	iTRAQ	ECM1	Limited patient samples	(Wang et al., 2017c)

1.5.3.8 Proteomics-based biomarkers for BDC

The most promising outcomes of proteomics have been accomplished in BDC or urinary cancer. Even though, cystoscopy technique is still the gold standard for early diagnosis of BDC worldwide (Gogalic et al., 2015). So far, proteomics based diagnostic markers BTA and NMP2 have been approved by FDA for clinical application (Chakraborty et al., 2019, Kim et al., 2020, Wilson Jr et al., 2020), the sensitivity could not reached to the up-to-mark level yet. However, researchers are working with non-FDA approved urinary proteins such as AURKA, BLCA-1, BLCA-4, COL1A1, FDCA, KRT8, KRT18, KRT20, PGRMC1, and ribitol, and tissue proteins such as BLCAP, CAIX, CCND1, CSTB, H2B, LSD1-AR, NIF-1, mapsin, p21, PFN1, PLK, and TP53 to improve and validate the biomarker list and to establish more sensitive, specific and potential non-invasive diagnostic biomarkers for BDC (Frantzi and Vlahou, 2017, Latosinska et al., 2018, Maas et al., 2018, Chakraborty et al., 2019, Kim et al., 2020, Wilson Jr et al., 2020). The proteomics analysis of BDC is abundantly heterogeneous. The advancement of analysis and peptides characterization will not only provide higher accuracy but also help us for better understanding of diseases, accurate personalized therapy, and cost effective diagnosis for all (Table 1.9) (Wilson Jr et al., 2020).

Table 1.9. Proteomics-based biomarkers for BDC

Sample sources	Methods	Proposed proteomics-based biomarkers	Remarks	References
Tissues	LC-MS/MS	EIF3D	Limited patient samples	(Latosinska et al., 2018)
Urine and cell lines	LC-MRM/MS	HSPE1	Absence of staging details	(Tsai et al., 2018a)

1.5.3.9 Proteomics-based biomarkers for other cancers

Minor changes of proteomes and metabolomes detection by powerful MS techniques can be used to screen and establish MS-based biomarkers in other cancers such as glioma, leukaemia, NPC, OVC, PNC, and testicular cancer. The high resolution peak, strong analytical power, peak overlapping error, higher acquisition, and ion based dissociation enabled MS to characterized phosphorylated and glycosylated peptides precisely (Cho, 2017). At the same time, heterogeneity and analytical power in clinical settings are major concerns. To overcome the difficulties related to the MS, advancement of instrumentation designed, powerful separation and data collection speed are monitoring regularly (Wentz and Danell, 2017, Jones et al., 2019). As a result, development and implementation of tendem, real-time mass defect filtering, and powerful detection of metabolites from two-phase system improved MS detection sensitivity from difficult-complex analytes and enabled them to provide structural baselines of the proteins (Cho, 2017, Wentz and Danell, 2017, Jones et al., 2019). Without a doubt, the recent advancement of MS technology and its implementation can be used as biomarkers for various cancers in clinical setting. For instance, MALDI-TOF, SELDI-TOF, iTRAQ and *in vitro* multivariate diagnostic assay-index have been approved for commercial use which identified OVA1 and ROMA as diagnostic biomarkers of surgery for women in OVC patients (Zhang et al., 2004, Dayyani et al., 2016).

1.6 Chapter Summary

CRC is a global threat affecting nearly two millions people worldwide (Ferlay et al., 2019). In this chapter, we have discussed the prevalence of the disease and the importance of early diagnosis for the management of CRC (Chapter 1.1 to 1.4). Colonoscopy and detection of occult blood in stool are routinely being used for screening but researchers are looking for non-invasive, cost effective, highly sensitive, and selective method. In this context, the genomic approach and proteomic approach for identification of cancer biomarkers may fulfil the need. Therefore, we have reviewed the role of lncRNA, particularly the autophagy-modulating lncRNA, as cancer biomarkers (Chapter 1.5.1 to 1.5.2). We further summarized the recent development in the detection of novel proteomics-based cancer biomarkers (Chapter 1.5.3). These background information enriched our understanding of CRC biomarkers and allowed us to identify the research gap for formulating the experimental design for this project.

Chapter 2

Research hypotheses and aims

2.1 Knowledge gap

According to GLOBOCAN statistics 2018, CRC is the second most lethal cancer in the world. In Hong Kong, CRC is the most common cancer and second leading cause of cancer-related death. The number of cases are increasing not only from the developed countries but also the developing countries (Brenner and Chen, 2018), (Chapter 1.1). Usually, patients with CRC has no sign and symptoms during earlier stages. It appears as irregular bowel movement, bloody stool or bleeding from rectum, abdominal discomfort, weakness and severe weight loss in later stages CRC. Despite the launch of screening programmes involving FOBT and colonoscopy (Chapter 1.2) more than 50% of the newly diagnosed cases in Hong Kong are already in Stage II or above (Hong Kong Cancer Registry, 2020).

The management of CRC is dependent on the detection stages. According to Young et al (2014), the curability of early stages CRC is > 90% by surgical removal of polyps. However, if CRC has already metastasized to distant organs, the 5-year survival would drop to < 10%. The most used CRC treatments are surgery, chemotherapy, and RT (Chapter 1.3). Usually, combined treatments are prescribed for better treatment outcome (Chapter 1.4.1, and Chapter 1.4.2). Over the past years, significant progress has been made in targeted therapy which is developed based on our knowledge of oncogenic genes, proteins, and other biomolecules that regulate CRC cell growth and survival (Chapter 1.4.2.3). Despite of the significant improvement in CRC management, there is a need of early diagnostic and prognostic biomarkers. It is anticipated that the CRC biomarkers would also facilitate better understanding of CRC development, the risk factors associated with the

carcinogenesis, and the molecular pathways involved in CRC pathogenesis. Ultimately, the knowledge gain can empower scientists and clinicians to reduce CRC related death worldwide.

The technological advancement of RNA sequencing revealed that about 2% of human genome capable of making proteins and remaining 98% considered as non-coding transcriptome. According to GENCODE database (www.gencodegenes.org), ~16000 lncRNAs genes have been identified so far which encode for about 30000 different lncRNAs (Chapter 1.5.1.1). The genome-wide expression profiles (GWES) and in situ expression analysis revealed that some lncRNAs are expressed in cytoplasm and some exclusively in the nucleus. Over the past two decades, numerous research evidence supports their critical roles in the pathogenesis of cancer including, cell proliferation, migration, differentiation, invasion, immune response, autophagy and apoptosis (Chapter 1.5.1.4). Sometimes, they act as tumour suppressor or oncogenes in transcriptional or post-transcriptional levels. Importantly, previous studies suggested that abnormal expression profile of lncRNAs can be used not only as potential diagnostic and prognostic biomarkers but also novel therapeutic targets for cancer therapy (Chapter 1.5.1.4).

Autophagy is a highly conserved and critical regulatory process for cells to maintain homeostasis by lysosomal degradation of various proteins and damaged organelles. Dynamic roles of autophagy have been identified in cancers where it participates in cancer progression, prevention, as well as drug resistance mechanisms. In addition, autophagy maintains homeostasis of cancer microenvironment by providing nutritional supplement under starvation and hypoxic conditions (Chapter 1.5.2.1, Chapter 1.5.2.2). Advanced research has shown that lncRNAs regulate most of the cancers by means of controlling the autophagy process and modulating the transcriptional and post-transcriptional ATGs (Chapter 1.5.2.5). In CRC, the expressions and functions of autophagy-

modulating lncRNAs are poorly studied so far. Therefore, further research is needed to elucidate the potential roles and molecular pathways of lncRNAs for their use as CRC biomarkers.

RT is a very useful therapeutic options for more than 50% of all cancer types. It is also very useful for the treatment of CRC when prescribed together with chemotherapy or surgery. In addition to lncRNAs, proteins are also useful biomarkers especially in cancer (Section 1.5.3). With the advancement in high-throughput technologies for protein analysis, proteomics-based cancer biomarkers may also allow us to study more specific pathways for CRC carcinogenesis.

Despite of significant improvement of RT, response rates are different for individual patients due to heterogeneity (Meehan et al., 2020). Apart from this, RT resistance is experienced by many CRC patients leading to high mortality (Kim et al., 2018b). It is anticipated that RT-related cancer biomarkers may provide prognostic or predictive information about patient response to RT. The information may be used to provide personalized RT treatment planning and reduce RT-induced toxicity to provide better therapeutic outcomes.

2.2 Hypotheses and aims

Based on the background knowledge from Chapter 1, we hypothesize that:

(a) Abnormal expressions of autophagy-modulating lncRNAs are highly associated with CRC carcinogenesis and silencing of the lncRNAs may suppress CRC progression.
(b) The expression of specific protein biomarkers may be altered in CRC tumours with different RT responsiveness.

The first goal of this study was to determine the roles of autophagy-modulating lncRNAs as novel biomarkers in CRC cells *in vitro.* The second goal of this study was to determine the specific proteins that may predict the response of CRC tumours to RT *in vivo.*

To achieve the aims of this study, we have designed the project with the following objectives:

(i) To determine the roles of two autophagy-modulating lncRNAs, namely CASC9 and RAMS11, in CRC carcinogenesis.

(ii) To identify specific metabolic proteins in highly responsive CRC xenografts in mice by high-throughput proteomics.

2.3 Significance of the project

The mortality and morbidity of CRC is highly correlated with advanced stages metastatic CRC patients. Our present study focused on the development of autophagy-modulating lncRNAs biomarkers for CRC and identified their molecular pathways association with carcinogenesis. Furthermore, we silenced these lncRNAs and explored their functional roles in CRC progression. Our findings provided evidence that silencing of lncRNAs might be used to suppress CRC progression. The lncRNAs studied may be used as novel therapeutic targets for CRC management. From our present study, we also identified specific protein markers that may be used to predict the radio-responsiveness of CRC tumours. The expressions of our identified proteins are significantly reduced along with effective RT. Therefore, it could be used as potential RT biomarkers for CRC tumours. Further studies may provide information to guide us to provide personalized RT treatment planning to achieve superior therapeutic outcomes. This study would enhance our understanding of the radiobiology of CRC and add new insight to the scientific community.

Chapter 3

Materials and methods

3.1 *In vitro* study of autophagy-modulating lncRNAs

3.1.1 Public data mining and analysis

The differential expression of selected lncRNAs and genes in CRC and adjacent normal tissues were analysed from two publicly available dataset: The Cancer Genome Atlas (TCGA) (http://gepia.cancer-pku.cn/detail.php) programme under National Cancer Institute (NCI) and The Encyclopedia of RNA Interactomes (ENCORI) (Li et al., 2013). From TCGA- colon adenocarcinoma (COAD) analysis, there were 275 tumour samples compared with 349 adjacent normal tissues for the expression profile analysis. $P < 0.01$ and fold change Log2FC >2.00 were considered as cut-off values to plot the box plot. The overall survival of CRC was determined by using TCGA-COAD dataset with a median cut-off, 95% confidence interval, counting the number of transcript per million (TPM), and considering the hazards ratio (HR). Likewise, the selected lncRNAs expression was extracted from the ENCORI-COAD dataset containing 471 tumour samples and 41 normal tissues were used to generate the box plot. The log-rank $P < 0.05$, HR, and high/low expression number were used to plot the survival curve from ENCORI-Pan-Cancer (Li et al., 2013).

3.1.2 Cell lines and culture conditions

Human normal colon cell, CCD-112CoN, was acquired from American Type Culture Collection (ATCC), (Manassas, VA, USA) and human CRC HT-29-Red-Fluc cell was acquired from PerkinElmer, Inc. (Waltham, USA). In addition, three more human CRC cell lines, namely DLD-1, HCT-116 and SW480 were kindly provided by Professor Jun YU, Department of Medicine and

Therapeutics, Institute of Digestive Diseases, The Chinese University of Hong Kong. These cell lines were chosen based on their frequent use in the literature and their spectrum of EMT characteristics as summarised in Table 3.1.

The growth condition of CCD-112CoN cells were maintained with 10% fetal bovine serum (FBS), (Gibco, USA) in Eagle's minimum essential medium (EMEM, ATCC, Manassas, VA). Whereas, HT-29, DLD-1, HCT-116 and SW480 were cultured in Dulbecco's modified eagle medium (DMEM, Gibco, USA) with 10% FBS. Cell culture was maintained at 37°C in 5% CO_2 in 100% humidity.

Table 3.1. List of CRC cell lines used

Cell line	Tissue	Characteristics
CCD-112CoN	Colon normal	• Fibroblast in nature
HT-29	Colon	• epithelial cell type • well established glandular adenocarcinoma
HCT-116	Colon	• well established for therapeutic research and drug screenings
SW-480	Colon	• more mesenchymal cell type
DLD-1	Colon	• intermediate epithelial-mesenchymal status

3.1.3 Drugs and chemicals

To induce and inhibit autophagy in CRC cells, the cells were treated with Rapamycin (100 nM) and Chloroquine (50 μM) respectively. The autophagy drugs rapamycin (autophagy inducer) and

chloroquine (autophagy inhibitor) were purchased from Sigma-Aldrich (Germany) and were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) and Phosphate buffer (PBS) solution (Gibco, USA) respectively. The concentration used were titrated in preliminary experiments. Figure 3.1 represent the preliminary results of rapamycin and chloroquine dose selection.

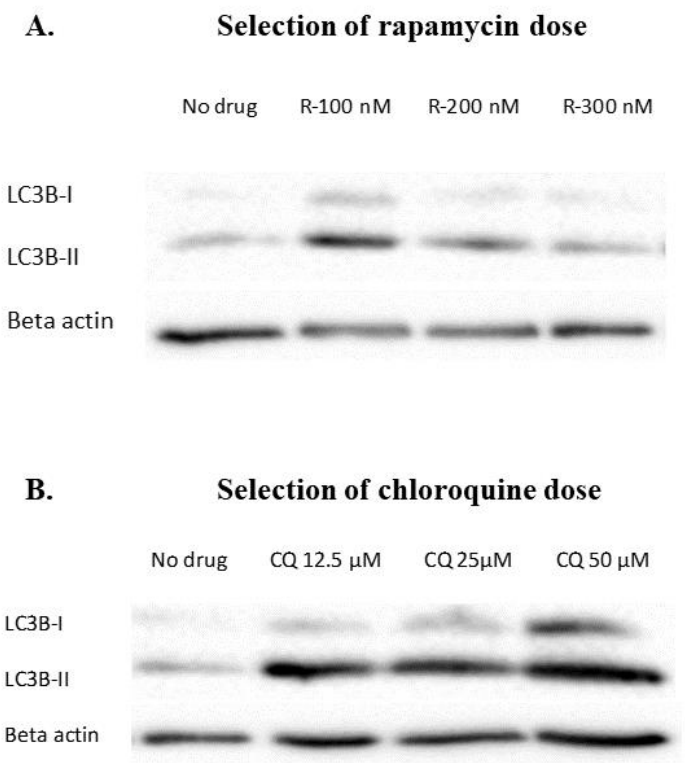


Figure 3.1. Selection of rapamycin (100 nM) and chloroquine (50 µM) doses. Based on literature studies, three doses of (A) rapamycin and (B) chloroquine were applied to the cells. The rapamycin dose 100 nM and chloroquine 50 µM were respectively selected by analysing autophagy marker LC3B compared to housekeeping gene beta actin.

3.1.4 RNA isolation and quality measurement

To extract the RNA from CRC cells, RNeasy Mini Kit (Qiagen, USA) was used and genomic DNA containment was removed by treating with DNase (Qiagen, USA). The quality of RNA was measured by using NanoDrop 2000 (ThermoFisher Scientific, USA), and Bioanalyzer respectively. The nano-spectrophotometric ratio of A260/280 and A260/230 more than 2.0 together with RNA integrity-valule (RIN-value) more than 9.0 were considered pure RNA. The RNA integrity was also checked by running total RNA into 1.2% agarose gel electrophoresis. The RNA quality was measured by agarose gel electrophoresis and bioanalyzer respectively (Figure 3.2).

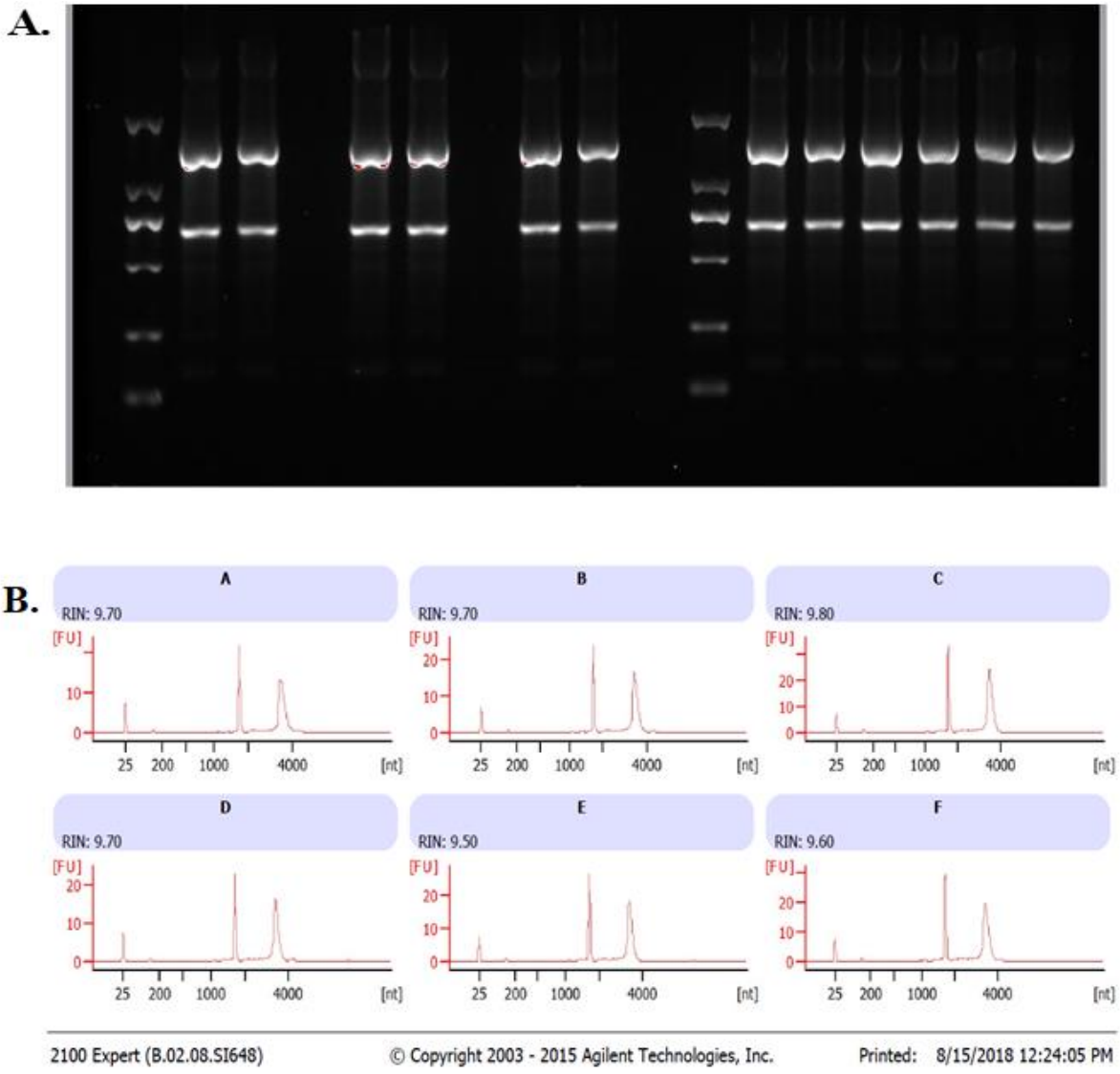


Figure 3.2. Quality determination of RNA sample. (A) The RNA quality was determined using 1.2% agarose gel electrophoresis, (B) The RNA-integrity was evaluated by bioanalyzer and it shown very high RIN-value.

3.1.5 Next Generation Sequencing (NGS)

The RNA-sequencing was performed by Centre for Genomic Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong. Briefly, KAPA Stranded mRNA-Seq Kit Illumina Platforms (Roche, Switzerland) was used to prepare the libraries using 1 µg of total RNA. The poly-T oligo containing magnetic beads was used to separate Poly-A containing mRNA from total RNA mixture which were then allowed to heat incubation (95°C) for about 5 minutes in the presence of magnesium to fragment them into 200-300 bp. The first-strand and second-strand complementary DNA (cDNA) were synthesized from fragmented parts by using reverse transcriptase enzyme and random-hexamer primer or oligo (dT) primers. The 10 cycled polymerase reaction was performed to enrich the library after adenylated and adaptor indexed ligation of double stranded cDNA. The libraries were then optimized and applied to Illumina NovaSeq 6000 to Pair-End 151 bp sequencing. The low quality sequence and more than 40 bp fragments were removed by filtration and final reads were compared with human reference genome using STAR (version 2.5). The expression quantification and differential gene expression were then performed by using RSEM (version 1.2.31) and EBSeq respectively. After several filtration using only lncRNAs and their fold change and FDR value, we identified 32 differentially expressed autophagy modulating lncRNAs from our dataset. They are alphabetically listed in Table 3.2.

Table 3.2. List of differentially expressed autophagy-modulating lncRNAs

LncRNAs name	Fold Change (Control Vs Rapa)	Regulation type	Fold Change (Control Vs CQ)	Regulation type
CASC11	6.29	Up	3.48	Up
CASC21	6.20	Down	5.48	Down
CASC9	8.86	Up	1.72	Down
CRCAL3/ LINC02163	1.58	Up	1.99	Down
DARS-AS1	4.37	Up	2.22	Down
DGCR5	2.18	Up	1.07	Up
DLX6-AS1	0	-	1.81	Up
FOXD3-AS1	1.26	Up	0	-
FRGCA	3.60	Down	1.0	Down
HRAT92	2.12	Up	3.18	Up
LINC00052	5.70	Up	5.48	Up
LINC00261	1.04	Down	1.18	Up
LINC00538	5.69	Up	3.24	Up
LINC00857	1.94	Down	1.42	Up
LINC00887	1.84	Up	1.87	Up
LINC00941	1.38	Up	2.29	Down
LINC01194	0	-	5.66	Down
LINC01559	2.04	Down	3.62	Up

Table 3.2. List of differentially expressed autophagy-modulating lncRNAs (Continued)

LSINCT5	3.60	Down	3.24	Down
MDC1-AS1	2.69	Down	3.24	Down
MIR155HG	8.80	Down	2.98	Down
MIR22HG	5.86	Down	2.22	Up
MYLK-AS1	5.20	Down	7.18	Down
NPPA-AS1	1.14	Down	7.90	Up
OLMALINC	3.85	Down	4.04	Up
PACERR	6.20	Down	5.48	Down
PCAT6	6.31	Down	4.91	Up
PRNCR1	4.41	Down	2.0	Down
RAMP2-AS1	3.31	Down	6.85	Up
RAMS11	-	-	8.86	Up
RASSF1-AS1	3.34	Up	5.48	Up
SATB2-AS1	3.60	Up	3.24	Down

3.1.6 Primer design

The autophagy regulated lncRNAs sequences were received from ensemble (Version100, 101), lncRNAdb (Version 15), and HUGO Gene Nomenclature Committee (HGNC). Primers were designed using NCBI primer-blast and IDT PrimerQuest Tool, the specific binding, hairpin formation and polymorphisms were avoided using NCBI Primer-blast, GENE TOOLS SNPCheck

(Version 3) and Oligo (Molecular Biology Insights, Inc., USA) respectively. All primers used in this study were purchased from Integrated DNA Technologies (IDT, USA) as listed in Table 3.1.

3.1.7 Complementary DNA synthesis and quantitative RT-PCR (qRT-PCR)

Following the standard protocol, first-strand cDNA was synthesized using Superscript II and Random Hexamer (Invitrogen, USA). Master Mix LightCycler 480 SYBR Green I (Roche, Switzerland) was used to complete the quantitative reaction using LightCycler 480 Instrument II (Roche, Switzerland). In order to get consistent results, melting temperature (Tm) 60±2°C and 45 cycles of amplification were followed. Detection of PCR product was based on SYBR green fluorescence signals. The melting curve analysis was performed to ensure specific target detection. Here, GAPDH was considered as the housekeeping gene and relative expression was calculated by $2^{-\triangle\triangle Ct}$ method.

Table 3.3. List of primers used in this study

Name of lncRNAs	Forward primer	Reverse primer
CASC11	ACCCTATGGAGAACCGAGAC	GAGGACCAACTCAGTAGGAAAT
CASC21	TCCAGAGGAGCCAAGAGAA	CAATGCTGTCCCACTCTGTATAA
CASC9	TTGGTCAGCCACATTCATGGT	GTGCCAATGACTCTCCAGC
CRCAL3	AAGGATGATACACATACATAGCC	TGAGACCAAGAACCCACA
DARS-AS1	AGCCAAGGACTGGTCTCTTTT	CTGTACTGGTGGGAAGAGCC
DGCR5	CTGAGCCCTATGACCCCAAC	GCTTGGTTCGCTTCTCCATC
DLX6-AS1	AGTTTCTCTCTAGATTGCCTT	ATTGACATGTTAGTGCCCTT
FOXD3-AS1	GAATAGTTGCCGAGAGAAA	GACAGACAGGGATTGGGTT
FRGCA	CGCAGCTAGGGAGCATTG	CTGAGGTGTATACTTGGGATGTG
GAPDH	TGCCATCAATGACCCCTTC	CATCGCCCCACTTGATTTTG
HRAT92	CTCATTCTTCGGCCAGTTATCC	CCGTCTTACCGAGTCCTCTAA
LINC00052	GCTCTCTCACCATGCGATT	TGTTTGCAGACTGTAGGGCT
LINC00261	GTCAGAAGGAAAGGCCGTGA	TGAGCCGAGATGAACAGGTG
LINC00538	TCAGGAGTCAGGAAGTCTGTAT	GTAACCGAGAGACTGGAAAGTG
LINC00857	CCCCTGCTTCATTGTTTCCC	AGCTTGTCCTTCTTGGGTACT
LINC00887	CTGCTCTGTGCCTGGTTATATT	CGCATCAGTTCTCTCTCATCTG
LINC00941	GACCTTTTCAGGCCAGCATT	ACAATCTGGATAGAGGGCTCA
LINC01194	AGACTGCTCTTGAGGCTGGAGT	AGGCTGAGGCTGGAGGATCTCT

- Supplier = IDT, USA

Table 3.3. List of primers used in this study (continued)

Name of lncRNAs	Forward Primer	Reverse Primer
LINC01559	GTCCTGCAGAACTCCCTCTT	AGTCCTGGAGCTGCAGAAAT
LSINCT5	GCTGGCCTTAGAACTGGATTAG	GTTCTTGATCTTCGGCAGGAA
MDC1-AS1	GATCCCAGATGTGCCAAAGT	AGCCAAGCTTCCATGGTTATC
MIR155HG	GAACAACCTACCAGAGACCTTAC	CACTCAGAGGATGAGGCATAAA
MIR22HG	AAGTTGGAGAGCCTTTGCCC	CGCACTATGGTGCCACATCT
MYLK-AS1	AGAGCAGGACAGCAGGTGTG	CCTGGCTTCCAATCTCACTG
NPPA-AS1	GTCCATGGTGCTGAAGTTTATTC	CCTCCAAGTCAGTGAGGTTTAT
OLMALINC	GACTCCTTTGGGAGACCAGTG	AGGTCACAGGGGATTTGATGG
PACERR	CTCCACGGGTCACCAATATAAA	ACGCATCAGGGAGAGAAATG
PCAT6	CCCCTCCTTACTCTTGGACAAC	GACCGAATGAGGATGGAGACAC
PRNCR1	CCAGATTCCAAGGGCTGATA	GATGTTTGGAGGCATCTGGT
RAMP2-AS1	GAACTCAGGCCAGATTTACAAG	TTGGGTCCTACAGCAACCAT
RAMS11	AAGAGGGCTAGAAGACGGGA	GGACACAGCTTTTGACGGTTC
RASSF1-AS1	ACCTATCTCAGTGGGTTACCT	TGCTAGGCGATAGAGATCCA
SATB2-AS1	TGAGAGCCCCATAACGGAA	CTGACCCAAGCAGAAACCCT

- Supplier = IDT, USA

3.1.8 Dicer-substrate mediated transfection

To knockdown CASC9 or RAMS11, Dicer-substrate mediated silencing was performed. HCT116 and SW480 cells were seeded and cultured in 6-well plate. Transfection experiment was performed when cell density reached 60-70% confluence. A lipid-based *in vitro* transfection was carried out by Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol. TriFECTa Kits were purchased from Integrated DNA Technologies (IDT, USA) which contained a Dicer-substrate negative control (DSi-NC), positive control (Dsi-HPRT-S1), transfection control (Dsi-TYE 563) and predesigned Dsi-RNAs (target genes) duplex. The duplex sequences for this study are listed in Table 3.2. The Dsi-NC and Dsi-HPRT-S1 sequence were not provided by the manufacturer. The transfection conditions were optimized in preliminary experiments.

Table 3.4. List of Dicer-substrate si-RNAs used in this study

Dicer substrate siRNA	**Duplex sequences**
Dsi-CASC9	5’ –GAGAGUCAUUGGCACUAUCAAGAAA- 3’, and 3’ –ACCUCUCAGUAACCGUGAUAGUUCUUU- 5’
Dsi-RAMS11	5’ –GAAUAAACAGGAUGUCUCUCACUTT- 3’, and 3’ –GACUUAUUUGUCCUACAGAGAGUGAAA- 5’

- Supplier = IDT, USA

3.1.9 Cell viability assay

After 24 hours of transfection, cells were trypsinized and counted by haemocytometer for seeding and performing cell proliferation assay using Cell Counting Kit-8 (CCK-8, Dojindo). 3-7 x 10^3 cells in 100µl of complete medium was seeded and cultured in a 96-well plate. According to CCK-8 cell proliferation assay protocol, 10 µL of CCK-8 solution was added to the well. After 3 hours incubation at 37°C + 5% CO_2, the amount of formazan which represents the number of live cells were measured at absorbance 450 nM using SPECTROstar Nano Microplate Reader (BMG Labtech, Germany).

3.1.10 Colony formation assay

Colony formation assay was performed to measure the cell proliferation *in vitro*. After being transfected for 24 hours, 1 x 10^3 cells were seeded and cultured for around two weeks in 6-well plate in triplicates. After colony formation, the colonies were fixed with a mixture of methanol and acetic acid at a ratio of 3:1. A solution of 0.5% crystal violet in methanol was used to stain and visualize the colonies. The images were photographed and the number of colonies were counted by ImageJ software (NIH).

3.1.11 Migration assay

In migration assay, 5-7 x 10^4 cells in 70 µl DMEM with 10% FBS were carefully placed in both compartments of the Culture-Insert 2 Well (Ibidi LLC, Germany). After 24 hours of cells settling, the culture inserts were gently removed by using tweezers to create a space of ~500 µm for measuring the cell migration ability. Then, each well was filled with 1.5ml of complete medium. The photographs of the wound areas were taken using an inverted microscope (Nikon, Japan) at

various time point of 0-hour, 24-hour and 48-hour respectively. The migration index indicating the size of the gap was measured using the MRI Wound Healing Tool in ImageJ (NIH).

3.1.12 Protein extraction

To extract protein from the cultured cells, culture medium was removed and cells were washed twice with 1x PBS. The cells were then collected and lysed using RIPA lysis and extraction buffer (Thermo Scientific, USA) on ice for 30 minutes with a supplement of cOmplete ULTRA Tablets, Mini EDTA-free, Easy pack Protease Inhibitor Cocktail (Roche, Switzerland). Centrifugation was performed to remove cellular debris and clear supernatant protein part was subsequently collected for further use. Furthermore, the protein concentration was quantified using BCA Protein Assay Kit (Thermo Fisher Scientific, USA).

3.1.13 Western blotting (WB)

The protein samples were gently mixed with 2x loading dye and heat incubation was performed for 5 minutes at 95°C to denature the proteins. A similar amount of proteins were loaded and run on 8-12% SDS-PAGE at ambient temperature. Proteins were then transferred onto Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Inc, USA), and followed by two hours blocking in 5% bovine serum albumin (BSA) (Hyclone BSA, GE Healthcare Life Science, USA) in Tris-buffer saline with a supplement of 0.1% tween 20 (TBST). Then the blocked membrane were incubated overnight with primary antibodies at 4°C. The membrane was then washed thrice (5 minutes each) with 0.1% TBST and the secondary anti-rabbit IgG, Horseradish peroxide (HRP)-linked or anti-mouse IgG-HRP-linked antibodies were added and incubated with the membrane for two hours. Afterwards, the membrane was washed thrice again with 0.1% TBST and Western Lightning Plus-Electrochemiluminescence (ECL) (PerkinElmer, Inc., USA) was added to the membrane

according to manufacturer's instruction to visualize protein bands in ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc, USA). The relative protein expressions were quantified using ImageJ software (NIH) with an internal control of β-actin or GAPDH. The primary and secondary antibodies used are summarized in Table 3.3 and Table 3.4 respectively. All antibodies except anti-PGK1 (Abcam, UK) were purchased from Cell Signaling Technology (CST, USA).

Table 3.5. List of primary antibodies used for WB

Name of the antibodies	Clone	Source	Catalog number
Acetyl-α-Tubulin	D20G3	Rabbit	5335
AKT		Rabbit	9272
AMPKα	D63G4	Rabbit	5832
β-Actin	D6A8	Rabbit	8457
Bcl-2 (Human specific)		Rabbit	2872
Bcl-xL	54H6	Rabbit	2764
Beclin-1		Rabbit	3738
Caspase-9 (Human Specific)		Rabbit	9502
E-Cadherin	24E10	Rabbit	3195
GAPDH	14C10	Rabbit	2118
GLUD1	D9F7P	Rabbit	12793
LC3B		Rabbit	2775
LDHA	C4B5	Rabbit	3582
mTOR		Rabbit	2972
N-Cadherin	D4R1H	Rabbit	13116
SQSTM1/p62		Rabbit	5114
PGK1		Rabbit	ab38007
Phospho-Akt (Ser473)		Rabbit	9271
Phospho-AMPKα (Thr172)		Rabbit	2535

Table 3.5. List of primary antibodies used for WB (continued)

Name of the antibodies	Clone	Source	Catalog number
Phospho-Akt (Ser473)		Rabbit	9271
Phospho-AMPKα (Thr172)		Rabbit	2535
Phospho-mTOR (Ser2448)		Rabbit	5536
Snail	C15D3	Rabbit	3879
Sox2	D6D9	Rabbit	3579
Vimentin	D21H3	Rabbit	5741

Table 3.6. List of secondary antibodies used for WB

Secondary antibodies	**Sources**	**Catalog number**
Anti-mouse IgG	Horse	7076
Anti-rabbit IgG	Goat	7074

3.1.14 Statistical analysis

All data are presented as mean ± standard error of mean (SEM) of at least three or more independent experiments. The statistical differences of the experimental data were calculated by student's t test or one way ANOVA using GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). The value of $P < 0.05$ is considered statistically significant.

3.2 Generation of mice xenograft CRC tumours *in vivo*

3.2.1 Ethical statement

The study was approved by the Ethics Committee of Centralized Animal Facility, The Hong Kong Polytechnic University. Animal experiment were obtained for all animals included in this study. The animal experiments were carried out in guidance with the licensing agreement of the Centralized Animal Facility, The Hong Kong Polytechnic University. There was no cruelty of animals during the experiments. A proper and decomposed procedure method were followed to kill the mice. This project was done in collaboration with Dr. Marco TAM, The Hong Kong Polytechnic University. I worked with Dr. TAM in all the animal experiments and Dr. TAM performed the volumetric analysis and determined the radioresponsiveness. The project ASESC No 18-19/69-HTI-R-GRF, Reference DH license No DH/SHS/8/2/4 Pt.7.

3.2.2 Mice model

Six to eight weeks old male nu/nu mice were purchased from the Laboratory Animal Services Centre of The Chinese University of Hong Kong. During the project, the mice were housed in Centralized Animal Facility, The Hong Kong Polytechnic University. The advantages of using male nu/nu mice are hairless and rapid growing of tumour cell lines due to absence of T cell or B cell and oestrogen disturbance. They were being kept in individually ventilated cages with

appropriate food and water supply. 12-hour dark and light cycles were maintained for the whole period of study. Before starting of the experiments, the mice were adopted with the living environment and sacrificed if they failed to thrive, such as drastic reduction of weight.

3.2.3 Tumour inoculation

Half million of HT-29-Red-Fluc cells were suspended in 100 µL of complete medium. The cell suspension was then injected subcutaneously. To increase the sample size and reduce the number of total mice used, cells were injected into both hind legs of mice. Immediately after tumour injection, the regular food was replaced with chow without alfafa and the normal water replaced by acidic water (pH 2.5-3). An antibiotic preparation of neomycin sulphate (Thermo Fisher Scientific, USA) was provided (dose 1.1 mg/ml) along with acidic water to reduce skin infection and acute post-irradiation gastrointestinal toxicity.

3.2.4 Measuring the tumour size

A high accuracy (0.1 mm) digital caliper was used to measure the mice tumour size. After visualization of prominent tumour growth, measurements of length and width (longer and shorter side) were counted two times a week and the tumour size was calculated by using the formula described by Sapi and colleagues (Sápi et al., 2015).

$$\text{Volume } (mm^3) = \text{Length (mm)} \times \text{Width}^2\ (mm^2) \div 2$$

3.2.5 Irradiation of tumours

After the tumour volume reached 100-300 mm^3 which was about 3-4 weeks post CRC inoculation, irradiation that mimicked RT treatment was conducted with 140 kV and 3 mA setting. The mice received a single postero-anterior field RT treatment of 15 Gy. The mice were anesthetized by

ketamine/xylazine during the RT treatments and lead shield was used to protect the body parts except irradiation site. To reduce the scattered dose of RT, a lead shield was also used beneath the mice. Unirradiated mice were used as control. After irradiation, the tumour size was similarly measured for 4 weeks to monitor the RT effectiveness. Moisturizing oil was used to treat the mice with acute skin reaction at the irradiation site. The RT response was defined by the reduction of tumour size from the initial irradiation treatments day to the last day of the experiments (Figure 3.3). After 4 weeks, the mice were sacrificed by applying an overdose of anaesthetics. The tumour actual size and metastatic natures were further verified after dissecting the tumour site and the tumour samples were preserved in -80°C for further analysis.

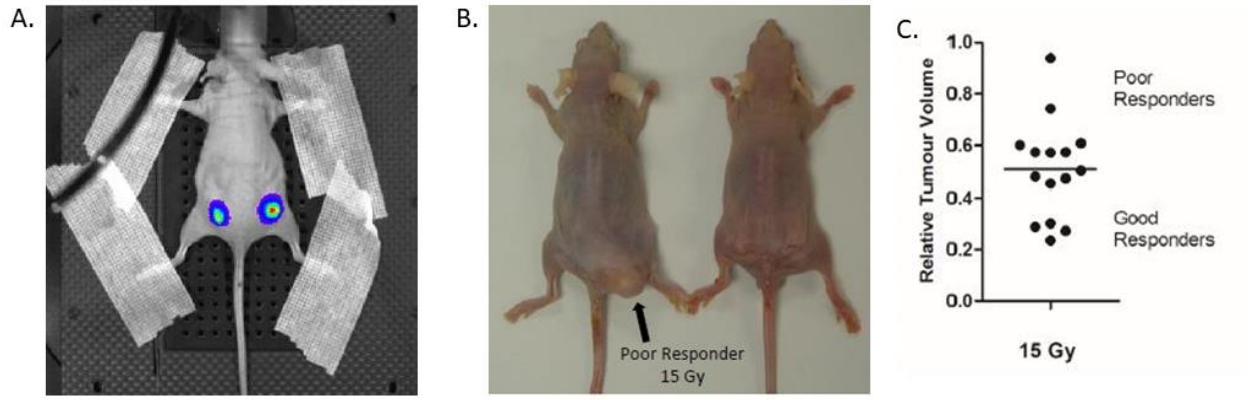


Figure 3.3. Tumour xenograft models with irradiation protocols. (A) The CRC tumours can be detected by bioluminescence imaging or digital caliper measurement. (B) About 4-5 weeks after post irradiation, mice were sacrificed administrating an over-dose of anesthetics. As shown by the black arrow, tumour responsiveness against radiation therapy vary even the same mouse. (C) Tumours were collected from the post 15Gy irradiatiated mice were ranked according to their relative size to the original tumours.

3.2.6 Selection of tumours for proteomics

After collecting the tumour samples, we further ranked the samples into three groups based on shrinkage of tumours sized against RT. The groups consist of (1) control group (unirradiated), (2) poor RT response, and (3) good RT response. Higher tumour size reduction was considered as better response. At least three samples from each group were chosen for conducting the proteomics study.

3.3 Proteomics

3.3.1 Mass spectrometry sample preparation for proteomics

Mass spectrometry samples were prepared using EasyPep Mini MS Sample Prep Kit (Thermo Scientific, USA) by their provided protocol. Briefly, 5 mg of tissues were taken in a centrifuge tube and disrupted with tissue homogenizer after adding 100 µL lysis solution. Universal nuclease was added along with lysis solution for complete digestion of nucleic acid contents in the mixtures. The purified proteins were collected after centrifugation and concentrations were measured using Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Then, 50 µg of proteins were transferred in a new tube and final volume adjusted to 100 µL with lysis solution. Afterwards, reduction and alkylation were performed by using reduction solution and alkylation solution. To block the reduction and alkylation, 10 minutes of heat incubation was performed. For digestion, reconstituted enzyme solution (Thermo Scientific, USA) was added to the preparation and incubated at 37°C for overnight and digestion was stopped by adding Digestion stop solution (Thermo Scientific, USA). Thereafter, the peptide solutions were desalted and cleaned by using a series of washing in a C18 Peptide Clean-up column. Lastly, the cleaned peptides were collected

by 70% acetonitrile in water with 0.1% trifluoroacetic acid elution and were dried by Refrigerated CentriVap Centrifugal Concentrator (Labconco Corporation, USA).

3.3.2 LC-MS/MS analysis

Proteomics experiments were performed in The University Research Facility in Chemical and Environmental Analysis, The Hong Kong Polytechnic University. The peptides fractioning and comparison were conducted on Orbitrap Fusion Lumos Mass Spectrometer. For the fractioning, Dionex Ultimate 3000 RSLCnano System decorated with Acclaim PepMap RSLC analytical columns (NanoViper, C18) (Thermo Fisher Scientific, USA) and Trap Column Cartridges Holders with nanoViper Fittings (Thermo Fisher Scientific, USA) were used. This unique combination provides high resolution separation, improved sensitivity in a constant flow rate 300 nL/min. The proteomics buffer concentration for all samples were maintained by 98% water with 2% acetonitrile and 0.1% formic acid. 1 µL of samples were injected for fractioning and collected peptides data from described experiments were analysed by Orbitrap Fusion Lumos Mass Spectrometer.

3.3.3 Data analysis

To complete the analysis, MS and MS/ MS peptides spectra were analysed by Progenesis QI for proteomics (Nonlinear Dynamics, Newcastle, UK) software compare with Swissprot dataset. The parameters for analysis were as follows: tolerance level → 10 ppm, maximum mass cleavage → 1, peptides charge: (2-4+), modification: both fixed and variable, Oxidation (M). The 1% False Discovery Rate (FDR) was applied to identify the peptides from complex mixtures. A fold change (FC) more than 2, $p = < 0.05$, minimum two unique peptide contents, and replicated proteins in all samples were counted to complete the analysis.

3.3.4 Bioinformatics analysis

Graphpad Prism Version 8.0.1 was used to generate the volcano plot data from raw proteomics. Heat map data was produced by Morpheus software (https://software.broadinstitute.org/morpheus/). Gene ontology (GO) and pathway data of significantly enriched protein of each group ($p \leq 0.05$, $FC \geq 2.0$) were generated by Enrichr (https://amp.pharm.mssm.edu/Enrichr/) functional analysis tool. Gene set enrichment analysis (GSEA) software (https://www.gsea-msigdb.org/gsea/index.jsp) was employed to identify the hallmark gene sets of the significantly enriched protein of each group. Web-based gene set enrichment analysis tool was used to generate rank-based pathway of proteins significantly deregulated between 15Gy good vs poor response (http://www.webgestalt.org/). Protein-protein interaction network of selected proteins were generated by STRING (https://string-db.org/) software.

Chapter 4

Cancer Susceptibility Candidate 9 (CASC9) Promotes Colorectal Cancer Carcinogenesis via mTOR-dependent Autophagy and Epithelial-mesenchymal Transition Pathways

4.1 Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy worldwide (Bray et al., 2018, Bray et al., 2020). Statistics revealed that in 2018, nearly 1.8 million new CRC cases were reported with ~0.9 million CRC deaths worldwide (Araghi et al., 2019). In the past decade, CRC treatment has progressed remarkably but late diagnosis and development of metastasis are the main obstacles leading to failure in CRC treatments (Islam Khan et al., 2019, Liu et al., 2020). Therefore, it is important to identify novel targets for early diagnosis and designing new therapy to minimize global CRC mortality.

Long-non coding RNAs (lncRNAs) are fragments of RNA that lack protein coding transcript. They are members of non-coding RNAs (ncRNAs). More specifically, LncRNAs contain more than 200 nucleotides, and are routinely transcribed by RNA polymerase-II in the human genome (Zampetaki et al., 2018, Islam Khan et al., 2019). In recent years, accumulating evidence has suggested that lncRNAs sometimes behave like regulatory molecules to control gene expressions. They are involved in the signaling pathways responsible for cell growth, development and metabolic processes (Lin and He, 2017, Sparber et al., 2019). In cancer, lncRNAs are associated with each stage of tumour initiation, progression, and poor prognosis by enabling drug resistance (Galamb et al., 2019, Qi et al., 2020). The aberrant expressions of lncRNAs alter the major oncogenic signaling cascades, for example WNT/B-catenin, P53, mTOR, PI3K/Akt, AMP activated protein

kinase (AMPK), EGFR, NOTCH, MAPK pathways (Kessler et al., 2013, Khan et al., 2015, Sever and Brugge, 2015).

In the past decade, many investigators concluded that abnormal expression of lncRNAs may be responsible for CRC inception, progression, and poor treatment outcomes of patients (Esmaeili et al., 2020, Qi et al., 2020). For instance, UNC5B antisense lncRNA 1 (UNC5B-AS1) has shown to reduced apoptosis to accelerate CRC progression and resulting in metastasis (Zhang et al., 2020c). Chen and colleagues illustrated that higher expression of KCNQ1OT1 promotes CRC carcinogenesis by enhancing aerobic glycolysis and stabilization of hexokinase 2 gene (Chen et al., 2020a). Likewise, FOXC2-AS1 promotes CRC progression via stabilizing FOXC2 and calcium channel controlled FAK signaling pathway (Pan and Xie, 2020). Bin and co-workers demonstrated that overexpression of EPB41L4A-AS1 is associated with CRC development. It activates Rho/Rho-associated protein kinase to promote CRC cell growth, proliferation and migration (Bin et al., 2021). Shan and colleagues revealed that Linc-POU3F3 acts as an oncogenic gene in CRC to promote initiation, progression and metastasis *in vitro* (Shan et al., 2016). In contrast, silencing of linc-POU3F3 reduced CRC carcinogenesis by inducing autophagy mediated apoptosis process (Shan et al., 2016). Another study shown that, TTN-AS1 silencing exerts its tumour suppressor activity through the suppression of epithelial-mesenchymal transition (EMT) process and PI3K/AKT/mTOR signaling (Cui et al., 2019). Overall, lncRNAs expression may promote or suppress tumours independently in each type of cancer.

Cancer susceptibility candidate 9 (CASC9), a recently discovered lncRNA, consists of four transcript variants CASC9-201, CASC9-202, CASC9-203 and CASC9-204. CASC9 earned significant attention of researchers due to the potential roles of its transcript variants in association with the pathogenesis of various cancers (Sharma et al., 2020). Recently, Luo and colleagues

revealed that upregulation of CASC9 is associated with advanced TNM stage and poor prognosis of CRC (Luo et al., 2019). In addition, CASC9 exerts its oncogenic activity through the phosphorylation of SMAD3 and TGF-β signaling *in vitro* (Luo et al., 2019). Another study performed by Ding et al. reported that CASC9 upregulation promotes CRC carcinogenesis by regulating miR-193a-5p and ERBB2 expression (Ding et al., 2020). Although these two studies demonstrated some roles of CASC9 in CRC, the molecular mechanisms of CASC9 in promoting carcinogenesis still remain largely unknown. Our present study aimed to explore the expression of CASC9 in CRC cell lines and to determine the role of CASC9 in mTOR dependent autophagy and EMT, which are associated with CRC progression. Our findings suggested that CASC9 might be used to evaluate CRC prognosis and it may be used as a novel therapeutic target for CRC patients.

4.2 Methods

To achieve the aim of this chapter, we first performed data mining and analysis (Chapter 3.1.1) followed by cell culture (Chapter 3.1.2), and RNA extraction (Chapter 3.1.4) to determine the expression of CASC9. Dicer-substrate medicated transfection was performed to knockdown the gene (Chapter 3.1.8). For exploring the role of CASC9 in different pathways, we performed cell viability (Chapter 3.1.9), colony formation assay (Chapter 3.1.10), migration assay (Chapter 3.1.11) and protein analysis (Chapter 3.1.12 and 3.1.13). Statistical analysis was performed as described in Chapter 3.1.14.

4.3 Results

4.3.1 CASC9 overexpression correlates with poor survival in CRC

To explore the role of CASC9 in CRC, we first searched the publicly available TCGA-COAD dataset (Figure 4.1.A). Box plot analysis of CASC9 showed that it was significantly upregulated in CRC samples (n = 275) compared with adjacent normal tissues (n = 349) (Figure 4.1.A). Furthermore, we evaluated the relationship between CASC9 expression and clinical outcomes of patients. To do so, we plotted the survival curve of CRC patients according to their CASC9 expression level, number of TPM, and hazard ratio (HR %) using TCGA-COAD dataset in Gene Expression Profiling Interactive Analysis (GEPIA) bioinformatics tool (http://gepia.cancer-pku.cn). We found that the patients with higher CASC9 have reduced overall survival but it did not reach statistical significance (Figure 4.1.B). To further confirm our findings, we explored CASC9 expression in another publicly available dataset, ENCORI-COAD (Figure 4.1.C). CASC9 expression was found to be overexpressed in 417 CRC tumour samples compared with 41 normal tissues (Li et al., 2013). Similarly, we plotted the survival curve for CRC patients based on log-rank $P < 0.05$, HR, and high/low expression profiles of CASC9 in the dataset. Higher expression of CASC9 from ENCORI-COAD dataset also showed a reduced overall survival of CRC patients compared to normal (Figure 4.1.D).

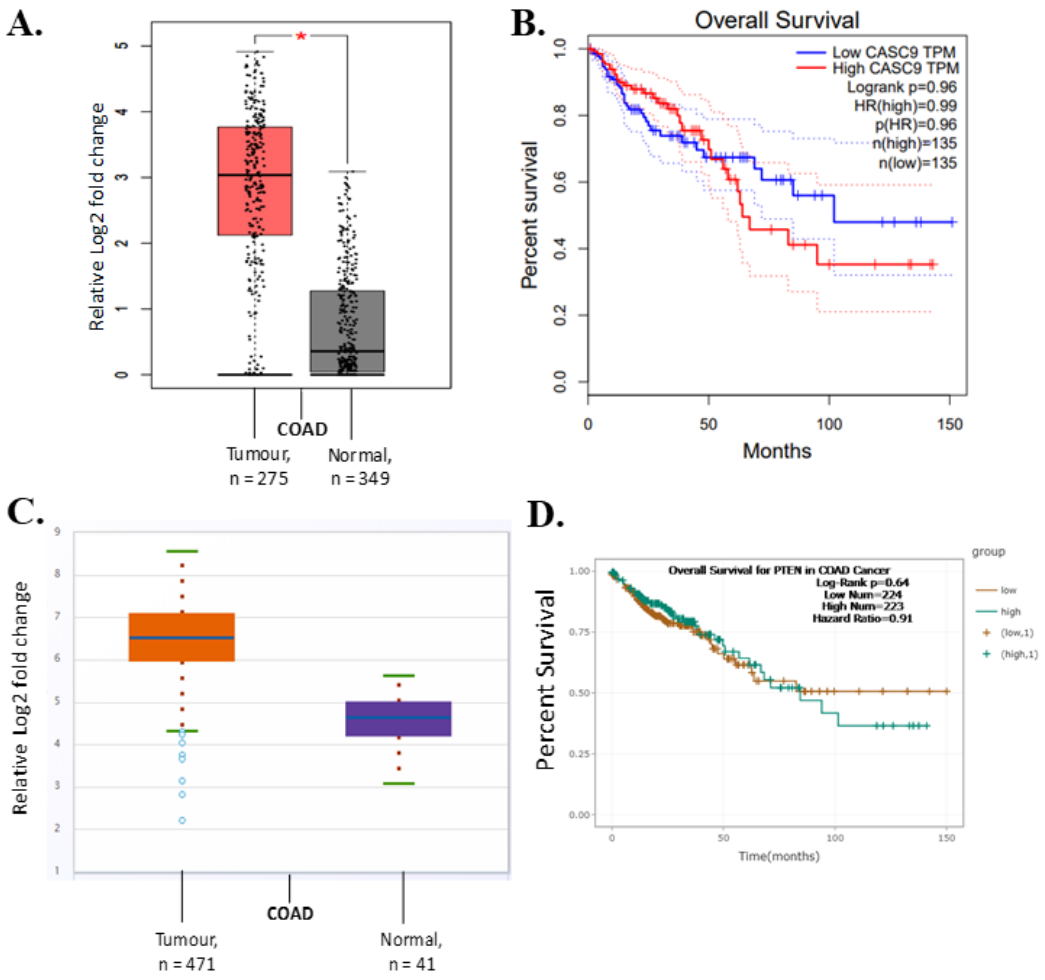


Figure 4.1. CASC9 overexpression is correlated with poor survival in CRC. (A) Boxplot CASC9 expressions of CRC tissues (n = 275) compared with normal samples (n = 349) from TCGA-COAD dataset (http://gepia.cancer-pku.cn/detail.php) showing that CASC9 was significantly upregulated in CRC tissues with a Log2FC cut-off value 2.0 and $P < 0.01$. (B) The expression profiles, number of TPM, and HR (%) were used to plot overall survival. Higher expression of CASC9 in CRC tissue is associated with poor overall survival. (C) CASC9 expression was extracted from ENCORI-COAD dataset (Li et al. 2013). 417 tumour samples and 41 normal tissues were used from the dataset. Boxplot analysis shown that CASC9 was upregulated in tumour tissues. (D) The log-rank $P < 0.05$, HR, and high/low expressions number

were used to plot survival curve from ENCORI-Pan-Cancer. Higher expression of CASC9 is related to the reduced overall survival of patients. (TCGA: The Cancer Genome Atlas, COAD: Colon adenocarcinoma, ENCORI: The Encyclopedia of RNA Interactomes, TPM: Transcript per million, HR: Hazard ratio).

4.3.2 CASC9 can be effectively and consistently silenced by Dicer-substrate siRNA techniques in CRC cells

CASC9 expression was measured in human CRC cell lines (DLD-1, HT-29, SW480 and HCT-116) and normal colon cell line CCD-112CoN (Figure 4.2.A) by RT-qPCR. Similar to the observation in public datasets, the expression of CASC9 was significantly upregulated in CRC cell lines compared to normal CCD-112CoN cells ($P < 0.001$, n = 8). The expression of CASC9 was low in DLD-1 and the highest level of CASC9 was detected in HCT-116 > SW480 > HT-29 cells (Figure 4.2.A). Therefore, HCT-116 and SW480 were chosen for gene silencing assay. The qRT-PCR results showed excellent knockdown efficiency of Dsi-CASC9 in HCT-116 and SW480 cells to be 63.25 ± 8.42 % and 58.0 ± 6.20 % respectively (Figure 4.2.B, C). To validate our knockdown method, the experiments were repeated on the gene of positive control (HPRT-1) in both cell lines and a knockdown efficiency of more than 60% was confirmed (Figure 4.2.B, C).

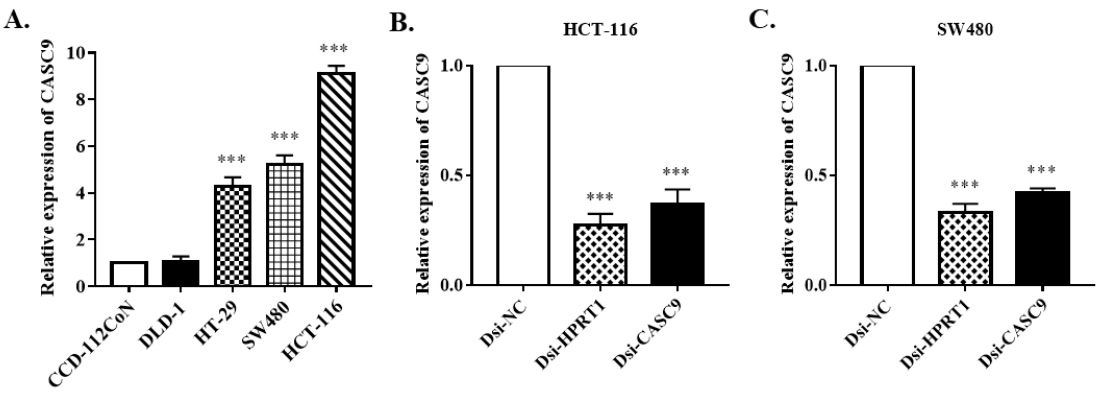


Figure 4.2. CASC9 expressions in colon cell lines and effective silencing by Dicer-substrate siRNA. (A) The expression of CASC9 in CRC cell lines DLD-1, HT-29, SW480, and HCT-116 was compared with normal colon cell line CCD-112CoN using RT-qPCR. The data is shown as mean ± SEM of 8 independent experiments. (B, C) Effective and consistent silencing of CASC9 by Dicer-substrate siRNA techniques was observed. The data is shown as mean ± SEM compared to the negative control Dsi-NC. (n = 6, *** $P < 0.001$).

4.3.3 Silencing of CASC9 reduced cell viability, colony formation and migratory index of CRC cells

To evaluate the biological and physiological functions of CASC9 in CRC cells, its expression in HCT-116 and SW480 cells were silenced by Dsi-CASC9 with Dsi-NC as negative control. By performing CCK-8 assay, we confirmed that CASC9 silencing significantly decreased the cell proliferation in HCT-116 and SW480 cells (Figure 4.3.A). Corresponding to cell proliferation, significantly reduced cell growth of HCT-116 and SW480 were evaluated after Dsi-CASC9 in colony formation assay (Figure 4.3.B). The migration assay was conducted in both cell lines to evaluate the migration ability of cells. Significant increase of migration index was shown in HCT-116 and SW480 after Dsi-CASC9 treatment (Figure 4.3.C) at 24 and 48 hours post transfection.

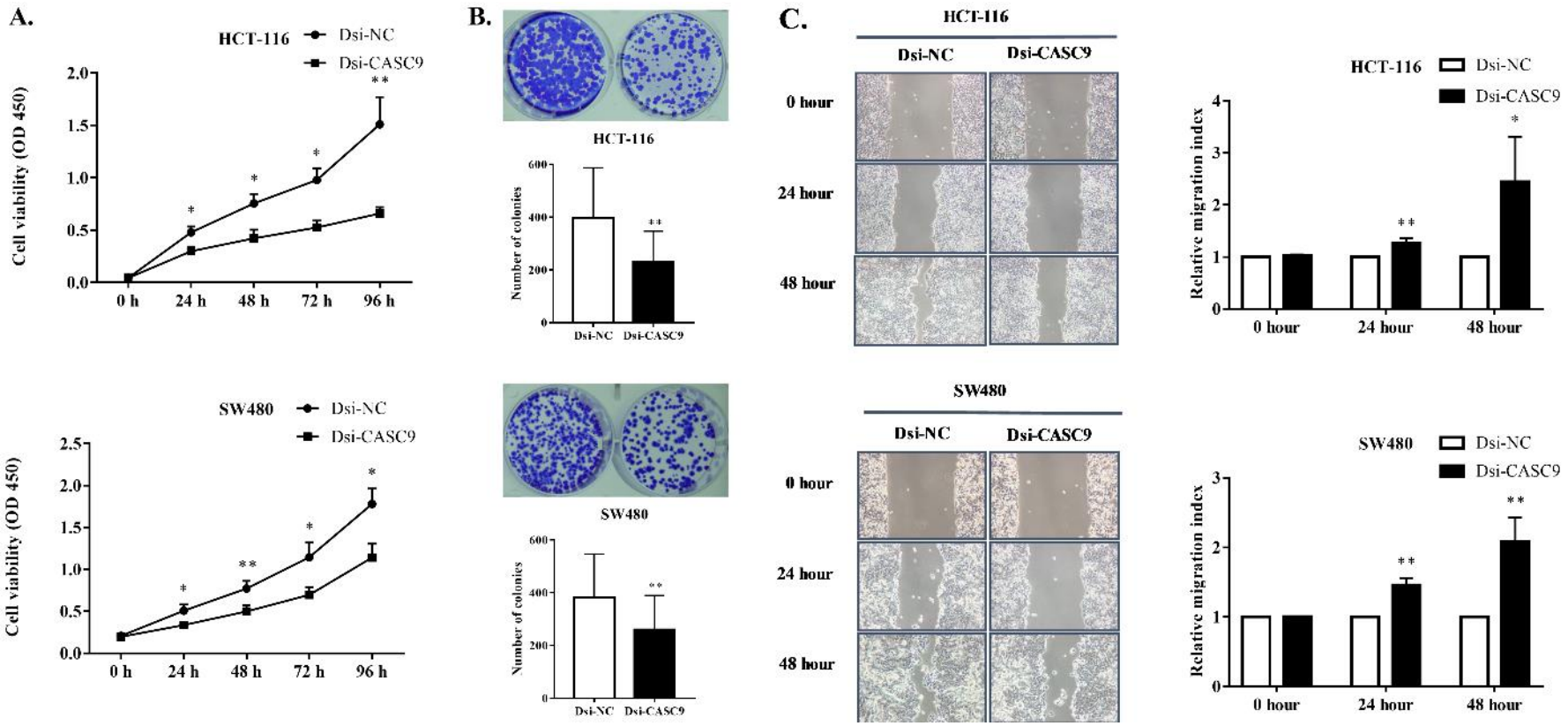


Figure 4.3. Silencing of CASC9 reduced cell viability, colony formation and increase migration index of CRC cells. (A) The silencing of CASC9 led to significant decrease in HCT-116 and SW480 cell proliferation (n = 4). (B) As determined by colony formation assay, the number of colonies in HCT-116 and SW480 were significantly reduced after CASC9 silencing (n = 4). (C) After knockdown of CASC9, the migration index of HCT-116 and SW480 was significantly increased at both the 24 and 48 hours timepoints (n = 6). Suggesting decrease in migration of cells to the gap. The data is shown as mean ± SEM compared to the negative control Dsi-NC. (*$P < 0.05$, **$P < 0.01$)

4.3.4 Silencing of CASC9 induced autophagy in CRC cells

Autophagy is a very crucial pathway for cell to survive during energy deficiency and hypoxic conditions. The WBt in (Figure 4.4.A, C) shown that the expression of LC3B-II (autophagy marker protein) was significantly upregulated in HCT-116 and SW480 cells after CASC9 silencing. Another autophagy marker protein p62, is a negative regulator of autophagy process. The silencing of CASC9 significantly reduced the expression of p62 protein level in both cell lines (Figure 4.4.B, D), suggesting that Dsi-CASC9 promote autophagy in HCT-116 and SW480 cells.

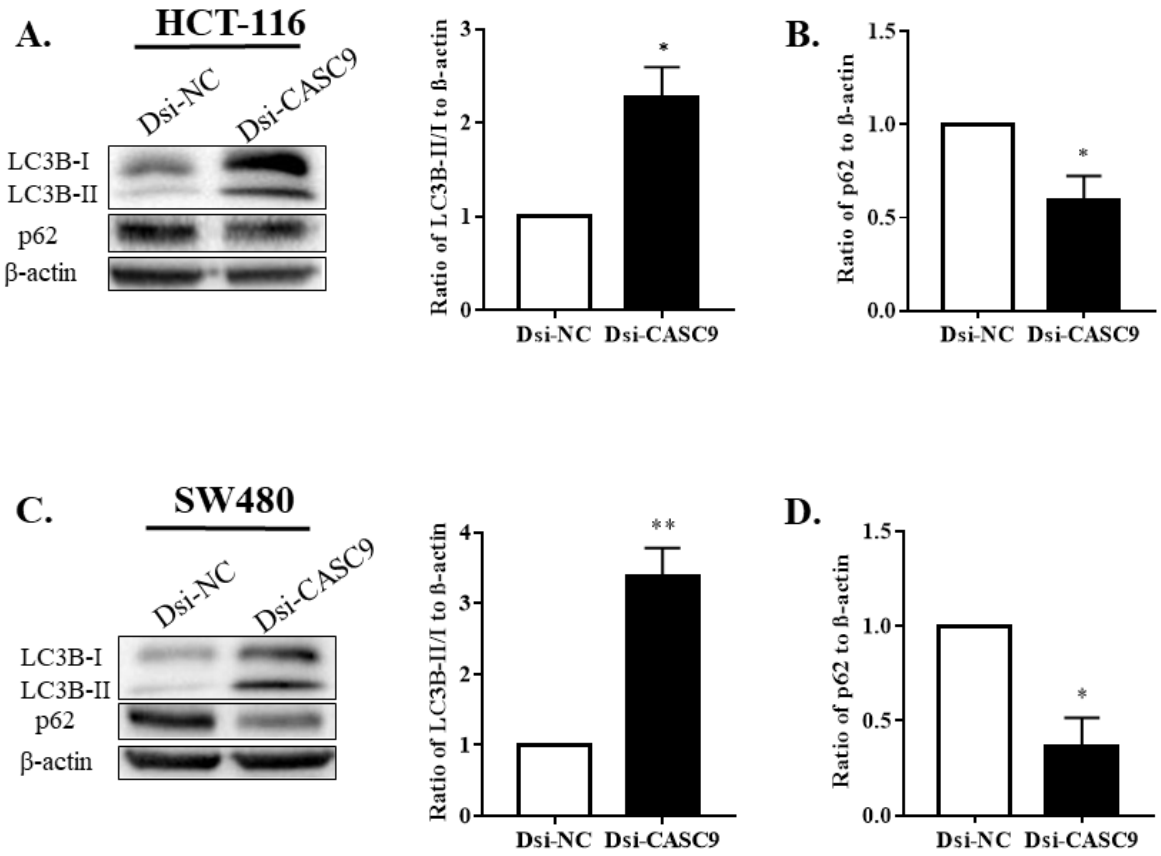


Figure 4.4. Silencing of CASC9 enhanced autophagy in CRC cells. The expressions of autophagy marker proteins LC3B and p62 were measured by WBtting in HCT-116 (A, B) and SW480 (C, D) cells. After Dsi-CASC9 mediated silencing, the ratio of autophagy marker LC3B-II to LC3B-I significantly increased in HCT-116 and SW480 cells with corresponding decrease in p62 expression. The data is shown as mean ± SEM using β-actin as housekeeping control. (*$P < 0.05$, **$P < 0.01$, and n = 4).

4.3.5 Silencing of CASC9 promoted the AMPK signaling pathway but downregulated the AKT/mTOR signaling pathway in CRC cells

We subsequently analysed more signaling pathway proteins to explore the role of CASC9 in CRC biology. The silencing of CASC9 promote the activity of AMPK and inhibit the AKT and mTOR pathway. To determine the regulation of mTOR pathway after silencing of CASC9, we investigated the upstream and downstream targets of mTOR. In this study, we found that Dsi-CASC9 significantly increased phosphorylation of AMPK compared to Dsi-NC in both HCT-116 and SW480 cells (Figure 4.5.A, B and E, F). On the other hand, AKT and mTOR were significantly downregulated in both cell lines (Figure 4.5.C, D and G, H) after CASC9 silencing.

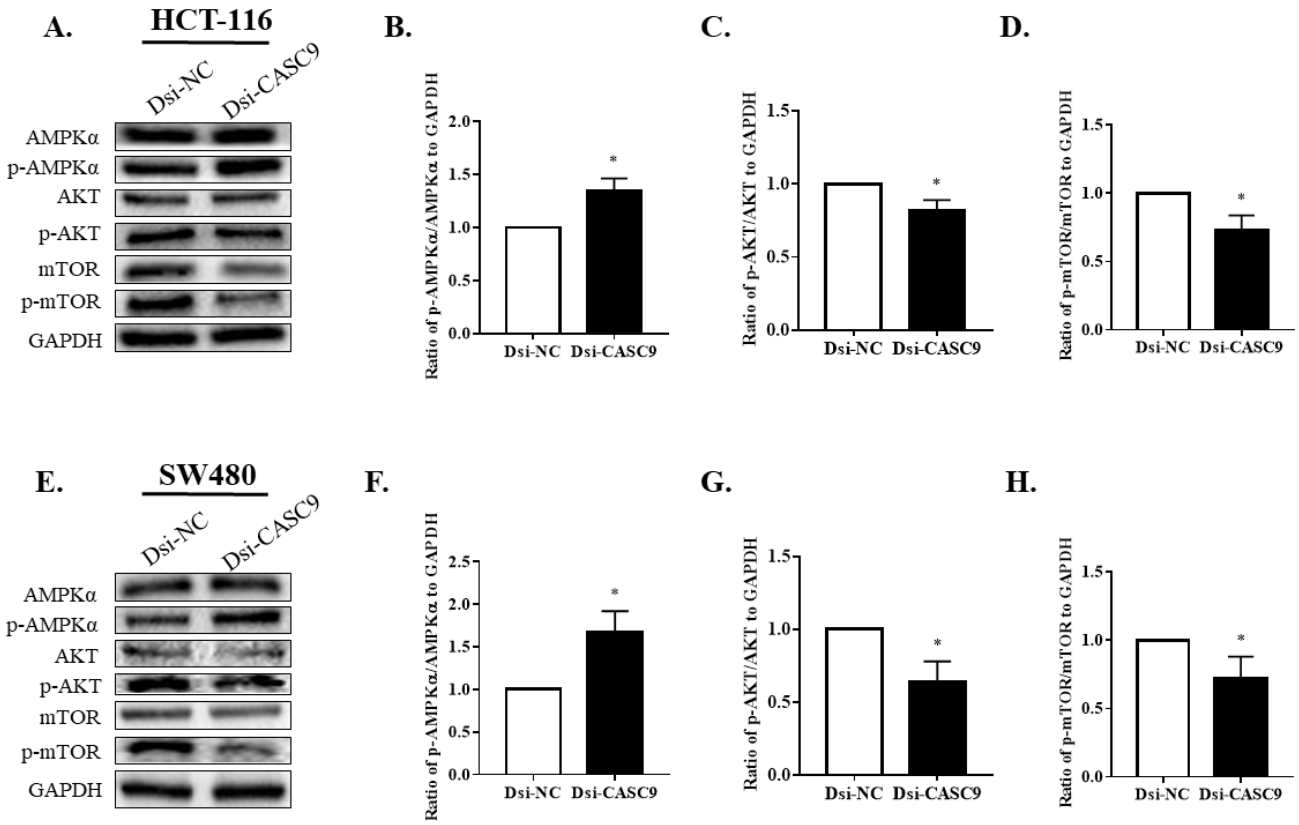


Figure 4.5. Silencing of CASC9 promoted the AMPK signaling pathway but downregulated the AKT and mTOR pathways. The ratio of p-AMPKα/AMPKα, p-AKT/AKT, and p-mTOR/mTOR to GAPDH were evaluated by WBtting in HCT-116 (A-D) and SW480 cells (E-H). Dsi-CASC9 significantly promotes AMPK signaling in HCT-116 and SW480 cells compared to Dsi-NC. In contrast, CASC9 silencing significantly downregulated AKT and mTOR signaling pathways in HCT-116 and SW480 cells. The data is shown as mean ± SEM using GAPDH as housekeeping gene. (*$P < 0.05$, and n = 3).

4.3.6 CASC9 silencing altered the expression of EMT marker proteins in CRC cells

EMT is one of the important steps of metastasis in cancer. We explored whether CASC9 silencing would alter the expression of key EMT regulatory proteins, such as E-cadherin, N-cadherin, and Vimentin, in HCT-116 and SW480 cells. As shown in Figure 4.6, the expression of epithelial marker E-cadherin was significantly upregulated in Dsi-CASC9 treated HCT-116 (Figure 4.6.A, B) and SW480 (Figure 4.6.E, F) cells. On the other hand, the mesenchymal marker protein Vimentin was significantly downregulated in HCT-116 and SW480 cells (Figure 4.6.D, H). The N-cadherin expression was also significantly downregulated in SW480 cell (Figure 4.6.G). However, the downregulation of N-cadherin in HCT-116 (Figure 4.6.C) did not reach statistical significance even though the trend was observed in all experiments.

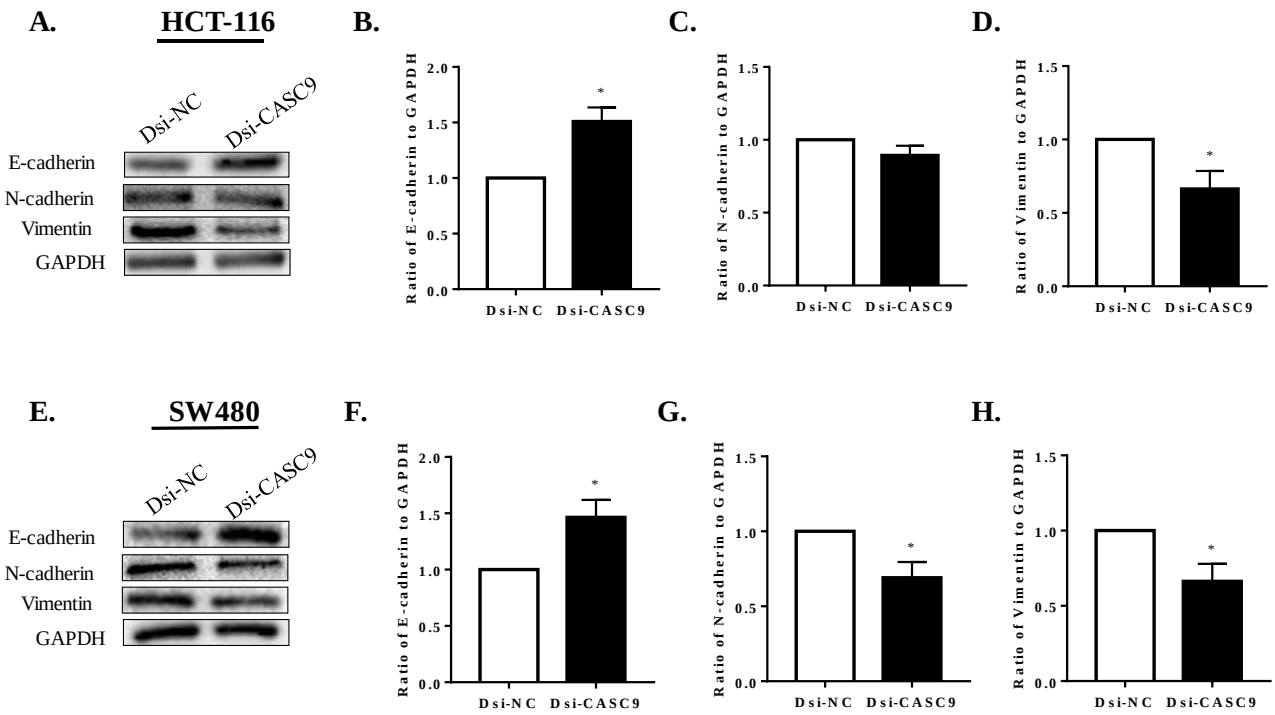


Figure 4.6. CASC9 silencing altered the expressions of EMT marker proteins in CRC cells. The EMT markers E-cadherin, N-cadherin, and Vimentin were evaluated by WBtting in HCT-116 (A-D) and SW480 cells (E-H). In both cell lines, CASC9 significantly upregulated E-cadherin and downregulated Vimentin expressions. The N-cadherin expression was also downregulated but did not reach statistical significance in HCT-116 cells. The data is shown as mean ± SEM using GAPDH as housekeeping gene. (*$P < 0.05$, and n = 3).

4.4 Discussion

With the advancement of next-generation sequencing technology, an increasing number of lncRNAs has been revealed. LncRNAs are not non-functional by-products or junk molecules of the body (Yao et al., 2019). They play very important roles in epigenetics and have multiple functions in cell growth and development. They are involved in various physiological process related to metabolism including gene mutation, regulation of transcription and translational processes, and regulation of cell cycles (Zhu et al., 2013). In carcinogenesis, lncRNAs play crucial roles in gene expression process by steering structural stability and transcriptional process of nucleus, regulating stability of mRNA, and maintaining transcriptional and post-transcriptional modification in the cytoplasm (Wilusz et al., 2009, Marchese et al., 2017, Zampetaki et al., 2018, Yao et al., 2019). So far, about 3000 lncRNAs have been identified from the human genome with their regulatory impacts on various cancer development, progression, metastasis, and poor prognosis (Gao et al., 2019).

The role of lncRNAs in CRC was firstly reported by Tsang and colleagues in 2010. They revealed that H19-derived miR-675 play an oncogenic roles in CRC development and progression by targeting retinoblastoma proteins (Tsang et al., 2010). Subsequent studies performed by Kogo et al. (Kogo et al., 2011) and Xu et al. (Xu et al., 2011) demonstrated that HOTAIR and MALAT1 are associated with poor prognosis of CRC by accelerating metastasis process. So far, a large number of lncRNAs has been reported as oncogenic or tumour-suppressors in CRC such as CCAT1, CCAT2, CDKNIA, GAS5, HOTAIR, MALAT1, MEG3, PANDAR, and UCA1. In addition, ncRuPAR and lincRNA-p21 are considered to be negative regulators of CRC in the process of radio-resistance and metastasis (Zhai et al., 2013, Yan et al., 2014).

In this present study, we examined the role of CASC9 in CRC. It is revealed that CASC9 is expressed in both normal and cancer cells but higher expression of CASC9 is associated with reduced overall survival and poor prognosis of patients. From our study, the expression of CASC9 was extracted from two publicly available databases TCGA-COAD and ENCORI-COAD. Based on the analysis of online databases, we observed that CASC9 expression was significantly upregulated in CRC tissues compared to its adjacent normal samples. Similar upregulation was also observed from ENCORI-COAD database analysis. Besides the expression analysis, we plotted overall survival curve and demonstrated that aberrant expression of CASC9 is strongly associated with reduced survival of patients. Furthermore, *in vitro* CASC9 expressions were determined and it was highly overexpressed in CRC cell lines compared to its normal cells. These findings suggested that CASC9 might be used as a novel marker for CRC prognosis. In fact, there are already many reports that CASC9 is involved in non-small-cell lung carcinoma, bladder cancer, thyroid cancer, hepatocellular carcinoma, nasopharyngeal cancer and lung cancer (Jin et al., 2019, Zeng et al., 2019, Chen et al., 2020b, Huo et al., 2020, Zhao et al., 2020). It has been suggested that CASC9 is a novel diagnostic, prognostic and therapeutic target in cancer treatment (Qian et al., 2020, Sharma et al., 2020). Our findings are in line with previous papers in CRC and other cancer (Luo et al., 2019, Ding et al., 2020), and hence, leading us to hypothesize that CASC9 may be involved in pathogenesis of CRC carcinogenesis.

Many studies have reported that CASC9 knockdown or silencing reduced cell proliferation, invasion and migration (Jin et al., 2019, Zhang et al., 2019a, Chen et al., 2020b, Fang et al., 2020, Huo et al., 2020). We are the first to perform similar experiments on CRC cell lines. By performing a series of *in vitro* experiments, including CCK-8 assay, colony formation assay, and migration assay after Dsi-CASC9 silencing, we confirmed that CASC9 played malignant roles in CRC cell survival, proliferation, and migration. To further decipher the role of CASC9

in CRC, we examined the potential pathways related to cell growth, apoptosis, and metastasis and decided to focus on autophagy and EMT (Vellai et al., 2008, Wang and Levine, 2010, Sever and Brugge, 2015, Mathiassen et al., 2017, Brabletz et al., 2018, Pavel et al., 2018, Pastushenko and Blanpain, 2019).

The self-degradation mechanism called autophagy is a major intracellular process that maintains the balance between cell death and survival in response to nutritional stress, hypoxia and growth factor deprivation (Tam et al., 2019, Noguchi et al., 2020). Autophagy is a dual-edged sword which can inhibit or promote carcinogenesis by regulating mTOR and apoptosis process (Levine, 2007). It is well established that lncRNAs promote or inhibit carcinogenesis by regulating autophagy either through mTOR dependent or independent pathways (Peng et al., 2020, Zhang et al., 2020a). To explore the autophagy process, we determined the expression of autophagy marker proteins LC3B and p62 before and after silencing. Dsi-CASC9 significantly increased LC3B-II and reduced p62 expression. The increased LC3-II is regarded as the standard marker for autophagy. It is directly associated with the number of autophagosomes and considered as the most commonly used autophagic marker protein (Zheng et al., 2012). The ubiquitin-associated protein p62 protein itself is degraded through autophagy and can also serves as a marker of autophagic flux. (Cohen-Kaplan et al., 2016, Liu et al., 2016a). Here, we clearly demonstrated the promotion of autophagy in CRC after silencing CASC9 (Mizushima, 2004, Jiang and Mizushima, 2015). We believe the induction of autophagy may be related to the reduced cell growth observed after gene silencing.

To further investigate molecular pathways in relation with reduced CRC cell proliferation and migration, we explored key signaling AMPK, mTOR, and AKT, which are linked to autophagy pathway. In mTOR dependent autophagy process, AMPK phosphorylates to activate upon energy starvation, leading to phosphorylation of Ser317, Ser777, and Ser555 to activate ULK1 and inhibition of mTORC1 signaling pathway (Paquette et al., 2018, Wang and Zhang, 2019).

The protein kinase B or AKT is one of the most critical intracellular pathways associated with mTOR signaling and it has been considered as the master regulator for most cancers (Porta et al., 2014, Yang et al., 2019). Inhibition of AKT/mTOR signaling promote autophagy and sensitize tumour cells to anticancer drugs by reducing cell growth, cell cycle, cell survival, differentiation and metabolism (Paquette et al., 2018, Terracciano et al., 2019). In this study, our key findings revealed that the silencing of CASC9 potentially promoted mTOR dependent autophagy where it significantly enhanced phosphorylation of AMPK and reduced phosphorylation of AKT and mTOR. The inhibition of AKT and mTOR pathways may lead to the attenuated cell growth and migration. Taken together, these findings revealed for the first time that abnormal expression of CASC9 promoted carcinogenesis of CRC cells through activating AKT/mTOR signaling, reduced phosphorylation of AMPK and inhibiting autophagy.

The poor prognosis for most cancers is due to development of metastasis where EMT enhances the cellular migration properties (Brabletz et al., 2018, Wang et al., 2020). In case of CRC, more than 20% of the patients were diagnosed when the tumours have already metastasized to distant organs (van der Geest et al., 2015). Our study revealed that CASC9 potentially induced EMT and silencing of CASC9 upregulated the epithelial marker protein E-cadherin and downregulated mesenchymal markers protein N-cadherin and Vimentin expressions. These results suggested that CASC9 may be involved in CRC progression and metastasis by regulating EMT-dependent migratory characteristics of CRC. Further study is needed to determine whether CASC9 may also be used as a therapeutic target. Targeted therapy with or without chemotherapy is mostly recommended to advanced stages CRC for successful eradicating of tumours and efficient patient care (Xie et al., 2020). The traditional targeted or immune therapy for cancer drug development mostly target the abnormal oncogenic proteins or stretches of DNAs. Recently, researcher are focusing in using lncRNAs as a novel set of therapeutic targets (Mitra and Chakrabarti, 2018). Theoretically, it may be possible to

downregulate CASC9 to reduce the chance of metastasis. However, we still need to work for the next leap of technology to reach this goal.

4.5 Conclusion

In conclusion, our findings demonstrated that CASC9 was aberrantly upregulated in CRC cells and tissues. We also revealed that silencing of CASC9 suppressed CRC proliferation, growth and migration via activation of mTOR dependent autophagy and EMT *in vitro*. Therefore, CASC9 may be an oncogenic regulator of CRC which promoted carcinogenesis via the suppression of autophagy and promotion of EMT. Our findings also suggested that CASC9 expression in tumour might be a potential biomarker and therapeutic target for the management of CRC.

Chapter 5

RAMS11 promotes CRC through mTOR-dependent inhibition of autophagy, suppression of apoptosis, and promotion of epithelial-mesenchymal transition

5.1 Introduction

In Chapter 1, we have already reviewed the background of CRC (Chapter 1.1). Indeed, CRC is one of the most commonly diagnosed malignancies, accounting second ranked in cancer-related death globally (Bray et al., 2018, Bray et al., 2020). Recently, in a study that compared the cancer site-specific and age-specific mortality in USA, it was reported that the incidence and lethality of CRC were increasing in young adults (Bhandari et al., 2017). Therefore, the search for potential biomarkers and therapeutic targets for CRC diagnosis and prognosis may be useful for identifying individuals at risk of development CRC or to hasten the diagnosis of early CRC for better treatment outcome. As elaborated in Chapter 1.2 and Chapter 4, lncRNAs have emerged to be a new aspect of cancer research. Strong research evidences suggested that their expressions are highly associated with specific cell-types and contributed to various cellular processes (Fang and Fullwood, 2016, Islam Khan et al., 2019). It is noted that lncRNAs bind with DNA or RNA or proteins and play gene mediatory roles by promoting or inhibiting the transcription process (Yang et al., 2015). Many studies also suggested that abnormal lncRNAs expressions contributed to CRC carcinogenesis through a cascade of regulatory signaling pathways, such as autophagy, EMT signaling, mTOR signaling, PI3K/AKT signaling, Wnt/β-catenin signaling, the Janus kinase/signal transducers and activator of transcription (JAK/STAT) signaling, MAPK signaling, p53 signaling, and Notch signaling, (Xu et al., 2014, Bermúdez et al., 2019, He et al., 2019, Tang et al., 2019, O'Brien et al., 2020). In addition, the expression of lncRNAs may be used to monitor CRC progression and may practicable as

diagnostic or therapeutic targets (Xu et al., 2014, Sun et al., 2018, Garcia et al., 2019). Therefore, exploring the epigenetic modification of lncRNAs associated with CRC growth and metastasis could open a new window for CRC diagnosis, prognosis and therapeutic targets.

RNA associated with metastasis-11 (RAMS11) is a newly identified lncRNA which was firstly identified by Dr. Maher's Lab (Silva-Fisher et al., 2020). Using meta-analysis, they discovered that RAMS11 is highly upregulated in metastatic CRC and associated with reduced disease-free survival. In addition, the *in vitro* results indicated that upregulation of RAMS11 promoted aggressive CRC phenotypes by increasing proliferation, migration, invasion, and number of colonies in CRC cells. Furthermore, RAMS11 knockout reduced CRC growth and metastasis *in vivo*. Although their study has reported the role of RAMS11 in CRC carcinogenesis, they did not explored the molecular mechanisms such as autophagy. In this chapter, we aimed to explore the RAMS11 expression in CRC cell lines and the in-depth mechanism associated with carcinogenesis.

5.2 Methods

The experimental design of this chapter is similar to that in Chapter 4. Except that data mining and analysis was not performed. We started off culturing the cells (Chapter 3.1.2) and extracted total RNA (Chapter 3.1.4) and determined the expression of RAMS11 by RT-qPCR (Chapter 3.1.7). Subsequently, we performed Dicer-substrate mediated transfection (Chapter 3.1.8) and explored the role of RAMS11 in cell viability (Chapter 3.1.9), colony forming (Chapter3.1.10), and migration (Chapter 3.1.11). We also performed protein analysis by WBtting (Chapter 3.1.13) and statistical analysis (Chapter 3.1.14).

5.3 Results

5.3.1 RAMS11 highly overexpressed in CRC cell lines and can be downregulated by Dicer-substrate siRNA techniques

The expression of RAMS11 was confirmed in CRC cell lines (DLD-1, HT-29, HCT-116, and SW480) and normal colon cells CCD-112CoN by RT-qPCR as shown in Figure 5.1. Our results indicated that RAMS11 was significantly overexpressed in CRC cell lines compared to the normal cell line CCD-112CoN (Figure 5.1.A). Similar to the expression of CASC9 in Chapter 4 (4.3.1), the overexpression of RAMS11 may be associated with the carcinogenesis of CRC. The most abundantly expressed RAMS11 was observed in SW480> HCT-116> HT-29>DLD-1. Therefore, SW480 and HCT-116 were selected for Dicer-substrate mediated gene knockdown assay. The knockdown efficacy and efficiency of Dsi-RAMS11 compared to Dicer-mediated negative control (Dsi-NC) was evaluated using RT-qPCR and we confirmed more than 70% silencing in both HCT-116 (Figure 5.1.B) and SW480 (Figure 5.1.C) cells.

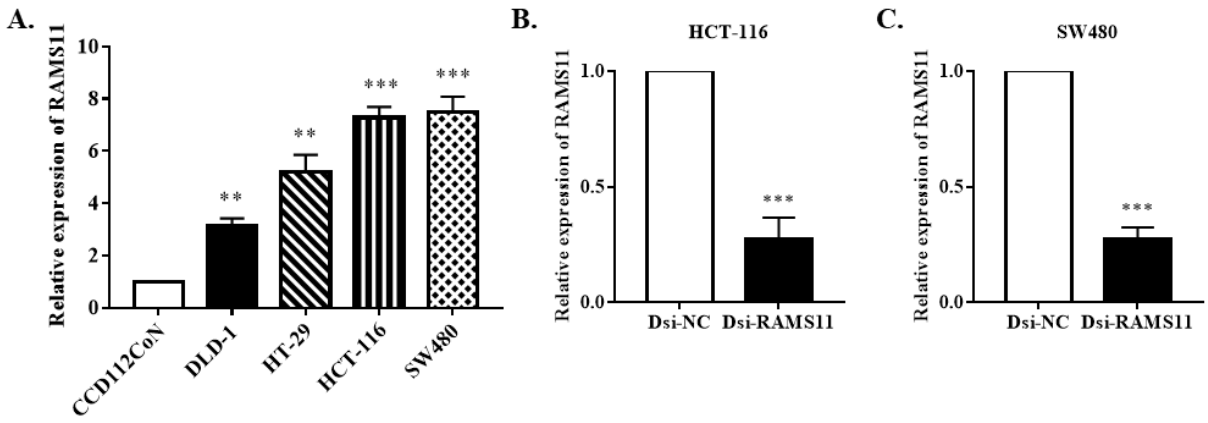


Figure 5.1. RAMS11 expression in colon cells and effective knockdown by Dicer-substrate siRNA techniques. (A) RAMS11 expression was measured in CRC cell lines (DLD-1, HT-29, HCT-16, and SW480) and colon normal cells (CCD-112-CoN) by using RT-qPCR. (B, C) The Dicer-substrate mediated RAMS11 knockdown was performed to downregulate the RAMS11 expression in HCT-116 and SW480 cells. The data were shown as mean ± SEM compared to normal cells, and negative control (Dsi-NC) group. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and n = 4)

5.3.2 Downregulation of RAMS11 inhibited cell proliferation, colony formation and migration of CRC cells

To understand the functional roles of RAMS11 in CRC proliferation, growth, and migration, we performed CCK-8 cell proliferation assay, colony formation assay, and migration assay. Our CCK-8 results showed that downregulation of RAMS11 significantly reduced cell proliferation of HCT-116 and SW480 cells compared to negative control Dsi-NC at 24, 48, 72, and 96 hours after Dsi-RAMS11 transfection (Figure 5.2.A). In concordance with the cell proliferation results, the colony formation assay showed that downregulation of RAMS11 significantly decreased the number of colonies in both HCT-116 and SW480 cells (Figure 5.2.B) compared to the Dsi-NC group. Next, the wound healing migration assay was performed to demonstrate the migration ability of HCT-116 and SW480 cells. Our results confirmed a significant higher migration index of HCT-116 and SW480 cells after Dsi-RAMS11 treatment at 24 hours and 48 hours post-transfection compared to negative control Dsi-NC (Figure 5.2.C).

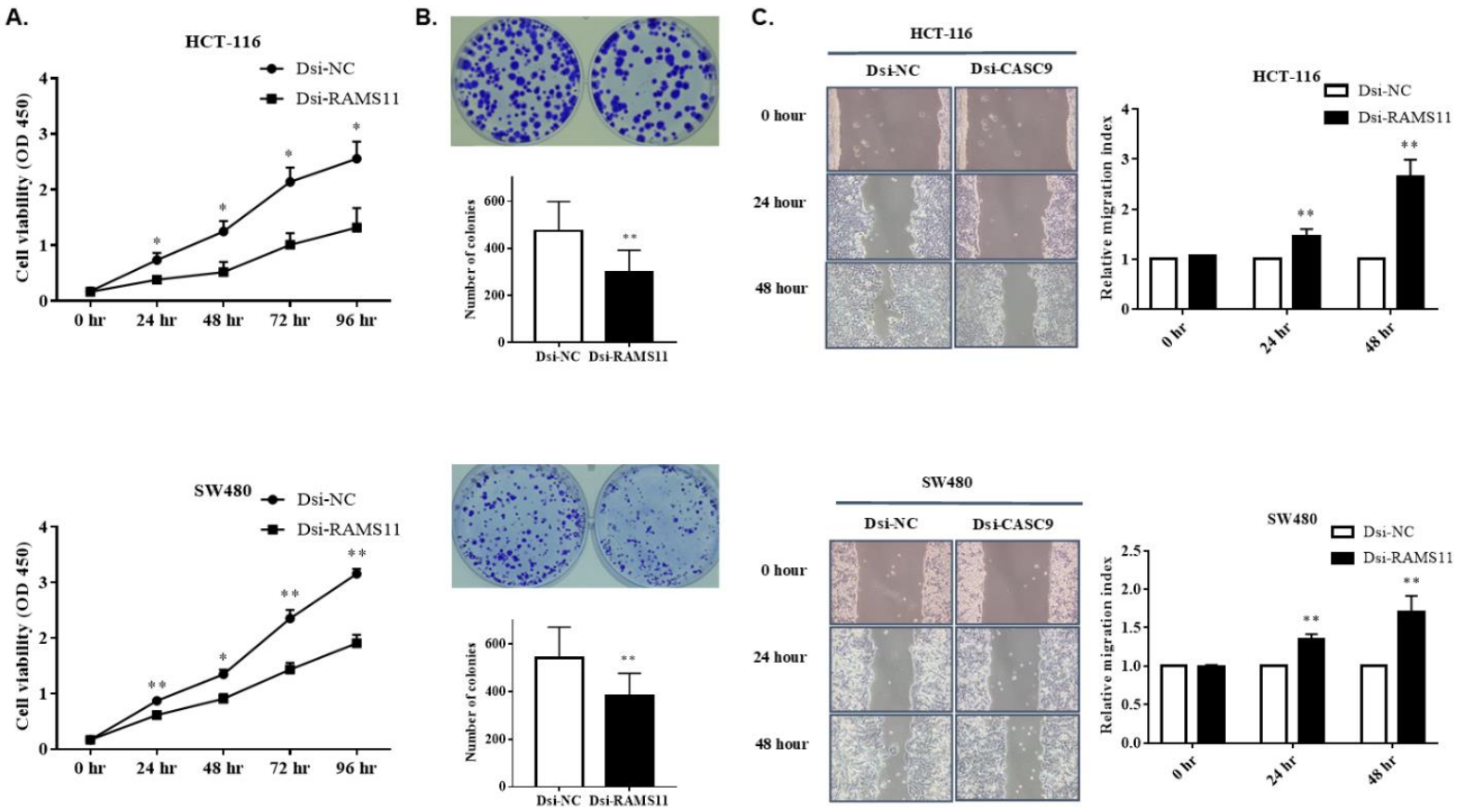


Figure 5.2. Downregulation of RAMS11 inhibited CRC cells proliferation, growth, and migration. (A) TCCK-8 assay was performed to evaluate the proliferation of HCT-116 and SW480 cells after Dsi-RAMS11 transfection. Downregulation of RAMS11 significantly reduced both cells proliferation at 24, 48, 72, and 96 hours of the experiments. (B) The number of colonies were also significantly decreased in both cells after Dsi-RAMS11. (C) The wound healing migration assay was performed to measure the migration index of HCT-116 and SW480 cells after Dsi-RAMS11. It shown that after RAMS11 knockdown, the migration index of HCT-116 and SW480 cells were significantly increased at 24 hours and 48 hours of experiments compared to Dsi-NC. The data was shown as mean ± SEM compared to Dsi-NC group. (*$P < 0.05$, **$P < 0.01$, and n = 4)

5.3.3 Downregulation of RAMS11 promoted autophagy in CRC cells

Autophagy is one of key regulatory self-degradative process of cells to maintain homeostasis, and survival during stress and hypoxic conditions. The autophagy levels in CRC cells HCT-116 and SW480 were evaluated by analysing autophagy marker proteins LC3B, p62, and Beclin-1 using WBt after Dsi-RAMS11 transfection (Figure 5.3). Our results demonstrated that downregulation of RAMS11 significantly increased LC3B expression in HCT-116 (Figure 5.3.A, B) and SW480 (Figure 5.3.E, F) cells. Silencing of RAMS11 significantly suppressed the expression of p62 in both HCT-116 (Figure 5.3.C) and SW480 (Figure 5.3.G) cells. Furthermore, we evaluated Beclin-1 expression which is associated with cellular key regulatory process autophagy and cell death. The WBt result showed that Dsi-RAMS11 significantly promoted Beclin-1 expression compared to Dsi-NC in HCT-116 (Figure 5.3.D) and SW480 (Figure 5.3.H) cells.

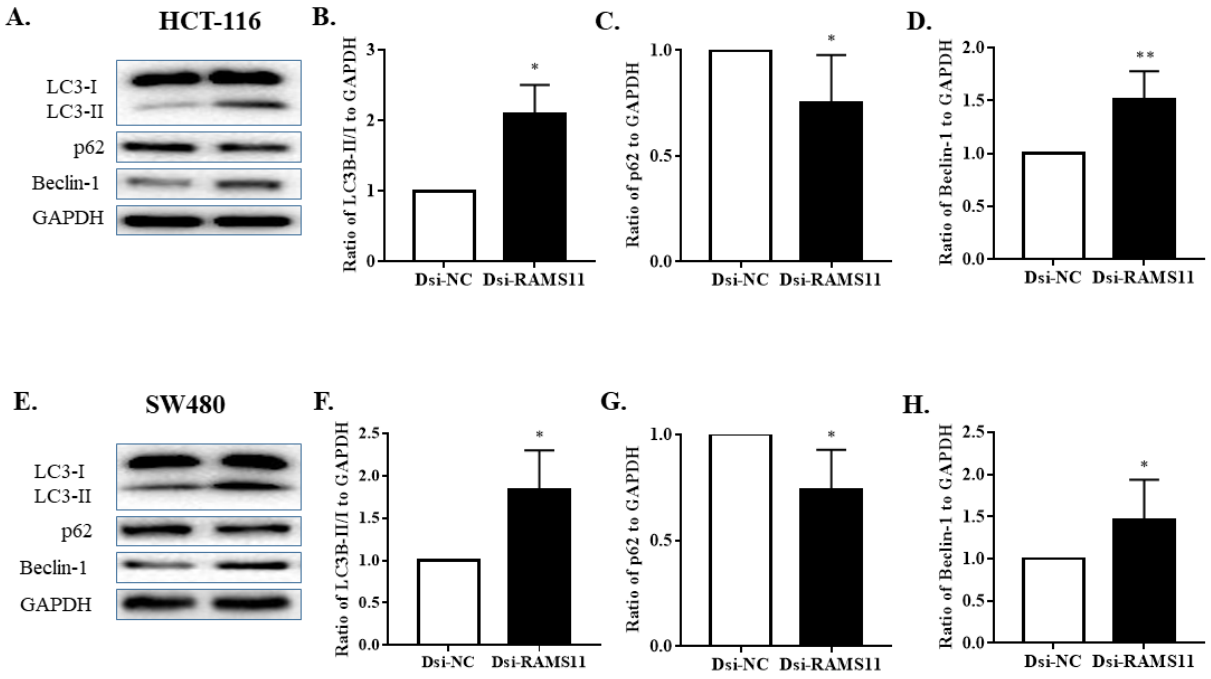


Figure 5.3. Downregulation of RAMS11 promoted autophagy in CRC cells. The autophagy marker proteins LC3B, p62, and Beclin-1 were measured using WBt in both HCT-116 (A-D), and SW480 (E-H) cells. The Dsi-RAMS11 treated cells increased ratio of LC3-II/ LC3-I, and Beclin-1 in both HCT-116 and SW480 cells, whereas reduced p62 expressions. The data was shown as relative expression mean ± SEM compared to Dsi-NC group using GAPDH as housekeeping gene. (*$P < 0.05$, **$P < 0.01$, and n = 4)

5.3.4 Downregulation of RAMS11 increased apoptosis of CRC cells

After confirmation of autophagy, we further explored the roles of RAMS11 in apoptosis (Figure 5.4). Bcl-2 regulates apoptosis by inhibiting apoptosis. Overexpression of Bcl-2 in cancer cells may block apoptosis and enhance cell survival. In our experiment the silencing of RAMS11 led to significant reduction of Bcl-2 in HCT-116 (Figure 5.4.A, B) and SW480 (Figure 5.4.E, F) cells. Similarly, another Bcl-2 family protein Bcl-xL was downregulated in HCT-116 (Figure 5.4.C) and SW480 (Figure 5.4.G) cells. Similarly, procaspase-9 expression was also reduced in both cells after Dsi-RAMS11 (Figure 5.4.D, H). These results suggested that knockdown of RAMS11 promotes apoptotic cell death.

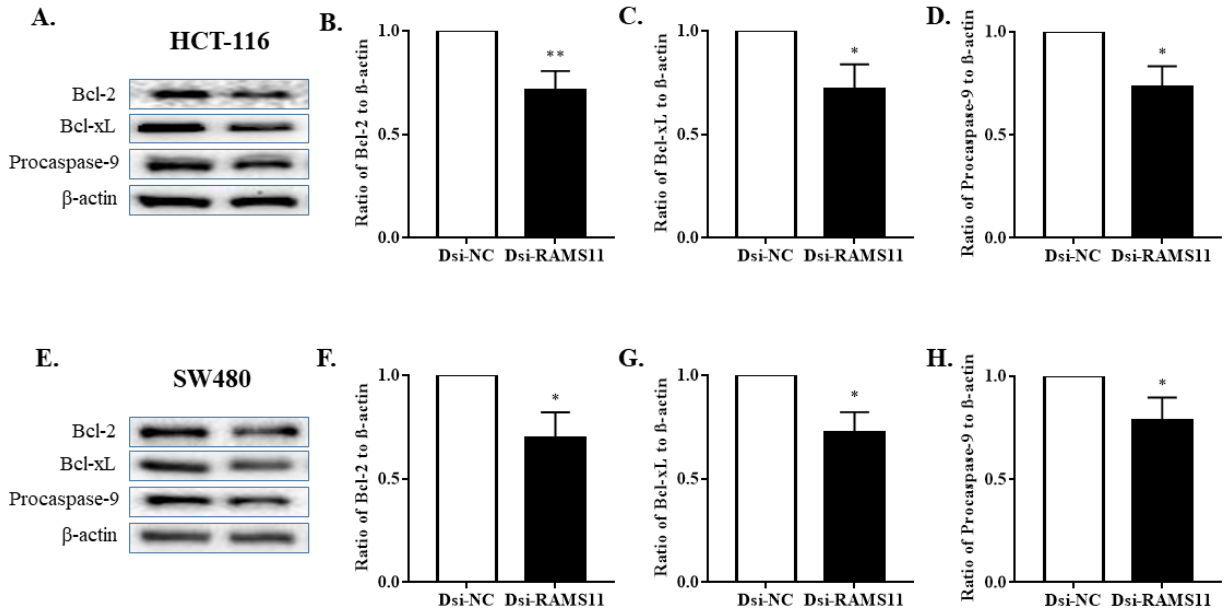


Figure 5.4. Downregulation of RAMS11 reduced the expression of apoptosis suppressors. The key apoptosis markers Bcl-2, Bcl-xL, and procaspase-9 were evaluated by WBtting in (A-D) HCT-116 and (E-H) SW480 cells. Downregulation of RAMS11 significantly reduced the expressions of Bcl-2, Bcl-xL, and procaspase-9 in both HCT-116 and SW480 cells compared to negative control Dsi-NC. The data was shown as relative expression mean ± SEM compared to Dsi-NC group using β-actin as housekeeping gene. (**P <0.05, **P <0.01*, and n = 4)

5.3.5 Downregulation of RAMS11 inhibited AKT/mTOR signaling via promoting AMPK signaling

Further investigation of the signaling pathways involving RAMS11 was performed by investigating the most frequently altered mTOR pathways with its upstream and downstream targets in CRC. To determine the AKT/AMPKα/mTOR signaling pathway, the phosphorylation of these proteins were analysed. As shown in Figure 5.5.A and E, the expressions of AKT, AMPKα, and mTOR in both Dsi-NC and Dsi-RAMS11 samples remained unchanged in both HCT-116 and SW480 cells. However, the expression of phosphorylated proteins p-AKT and p-mTOR expression in HCT-116 and SW480 cells were downregulated after Dsi-RAMS11 transfection. We also confirmed that Dsi-RAMS11 significantly increased the p-AMPK expression in both cells (Figure 5.5.C, G) compared to the Dsi-NC group. This results indicated that Dsi-RAMS11 may promote the activation of AMPK by reducing phosphorylation of p-AKT and p-mTOR.

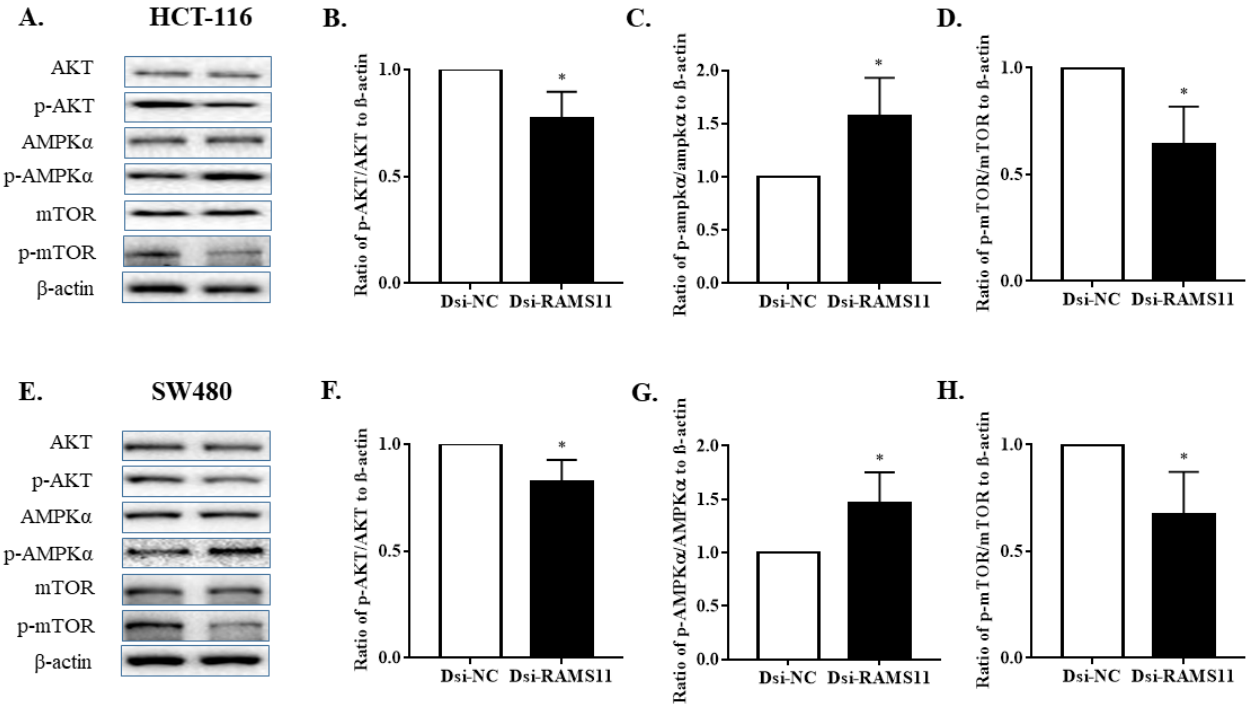


Figure 5.5. Knockdown of RAMS11 downregulated AKT/mTOR signaling by promoting AMPK pathway. Downregulation of RAMS11 significantly reduced the phosphorylation of AKT and mTOR and induced phosphorylation of AMPK in (B-D) HCT-116 and (F-H) SW480 cell. The data was shown as relative expression mean ± SEM compared to Dsi-NC group using β-actin as housekeeping gene. (*$P < 0.05$, and n = 4)

5.3.6 Downregulation of RAMS11 negatively altered EMT marker proteins and transcription factors

We subsequently evaluated the expression of EMT marker proteins in CRC cell lines and evaluated the effect of RAMS11 gene silencing. Cancer cells gain migratory characteristics resulting in development of metastasis, chemo-resistance, and immune-suppression via EMT pathways. In EMT, the epithelial marker E-cadherin level is decreased, and mesenchymal proteins and transcription factors N-cadherin, Vimentin, Sox2, and Snail levels are increased. In our study, we found that Dsi-RAMS11 significantly increased the epithelial marker E-cadherin in HCT-116 (Figure 5.6.B) and SW480 (Figure 5.6.H) cells compared to Dsi-NC. On the other hand, Dsi-RAMS11 significantly decreased the mesenchymal marker proteins N-cadherin and Vimentin in both cell lines HCT-116 (Figure 5.6.C, D) and SW480 (Figure 5.6.I, J). Apart from that, we also evaluated the expressions of EMT regulated transcription factors Sox2 and Snail. Our results showed significant reductions of Snail and Sox2 expressions after Dsi-RAMS11 treatment in HCT-116 (Figure 5.6.E, F) and SW480 (Figure 5.6.K, L) cells.

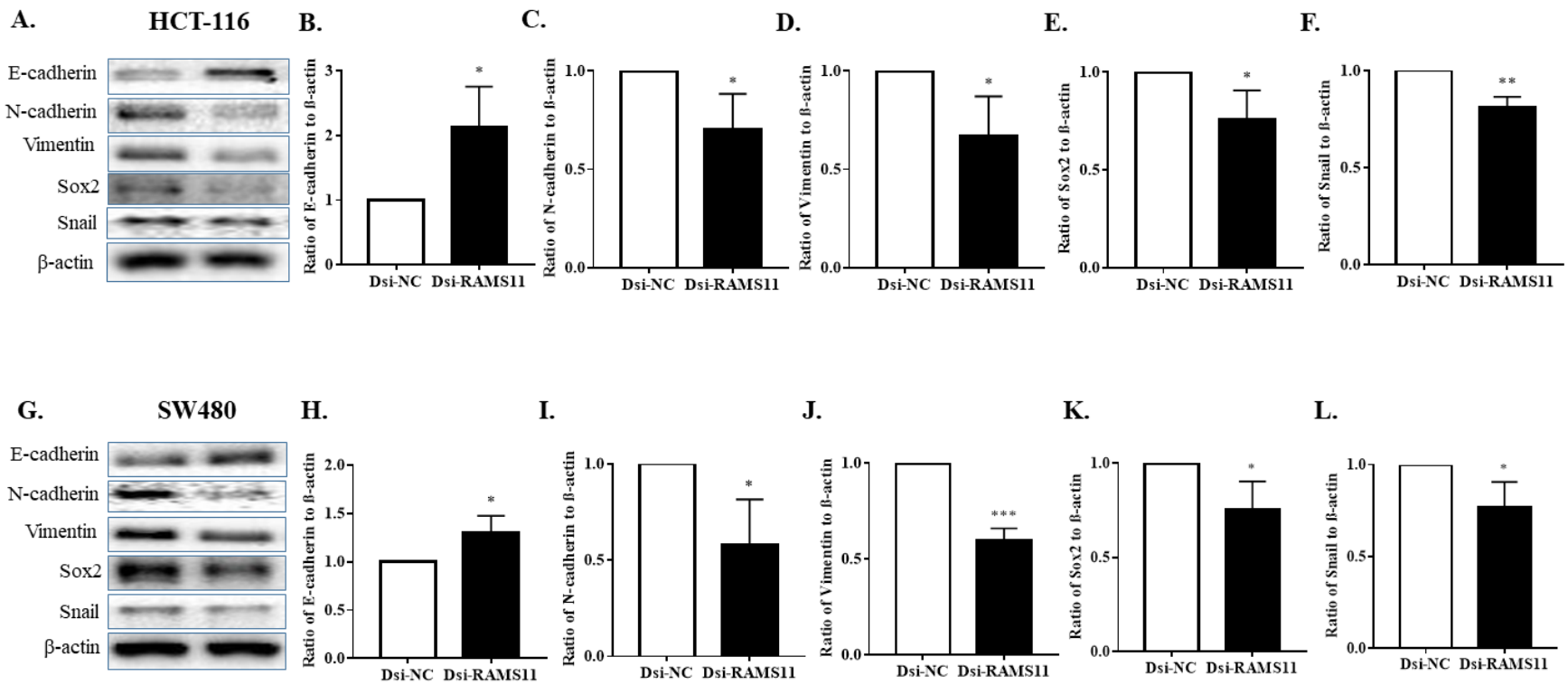


Figure 5.6. The knockdown of RAMS11 inhibited EMT markers and transcription factors in CRC cells. The EMT markers and transcription factors expressions were evaluated by WBtting in (A-F) HCT-116 and (G-L) SW480 cells. The Dsi-RAMS11 mediated knockdown significantly enhanced the expression of E-cadherin in both (B) HCT-116 and (H) SW480 cells compared to Dsi-NC. On the other hand, Dsi-RAMS11 significantly decreased mesenchymal markers N-cadherin and Vimentin expressions in (C-D) HCT-116 and (I-J) SW480 cells. In addition, EMT regulated transcription factors Sox2 and Snail expressions were significantly downregulated in (E-F) HCT-116 and (K-L) SW480 cells after Dsi-RAMS11. The data was shown as relative expression mean ± SEM compared to Dsi-NC group using β-actin as internal control. (**P <0.05*, ***P <0.01*, ****P <0.001* and n = 4)

5.4 Discussion

In this study, we are the first to demonstrate that lncRNA RAMS11 is associated with CRC progression and metastasis *in vitro*. Our study demonstrated that downregulation of RAMS11 is negatively associated with CRC cell proliferation, growth, and metastasis via autophagy, apoptosis, and AKT/AMPKα/mTOR signaling pathways.

Previous studies have reported that knockdown or downregulation of many carcinogenic genes or lncRNAs could potentially inhibit the tumour progressions (Song, 2007, Siddiqui et al., 2019). Hence, we knockdown RAMS11 and perform *in vitro* cellular functional assay like CCK-8 assay, colony formation assay, and migration assay. The functional assays after RAMS11 downregulation displayed significantly reduced CRC cell survival, proliferation, and migration. Our results are in line with previous studies that demonstrated knockdown or knockout of lncRNAs such as H19, SNHG14, CCAL, and MIR17HG (Ma et al., 2016b, Di et al., 2019, Xu et al., 2019, Zhan et al., 2020a, Zhang et al., 2020b) potentially reduced CRC cell proliferation, migration, metastasis, and increased chemo-sensitivity.

Our results are in line with previous publications. To further decipher the RAMS11 knockdown association with reduced CRC cell growth, proliferation, and migration, we evaluated the potential molecular pathways related to growth, apoptosis, and metastasis. Autophagy is the cellular lysosomal degradative process of removing unnecessary or folded materials to maintain homeostasis and restore energy during nutrient stress and hypoxic conditions (Eisenberg-Lerner et al., 2009). It is well established that inhibition of autophagy reduced elimination of damaged particles, accumulate folded materials from cells and results in cancer development (Yun and Lee, 2018). In our exploration, we evaluated autophagy marker proteins LC3B, p62, and Beclin-1 in Dsi-NC and Dsi-RAMS11. The downregulation of RAMS11 significantly promoted LC3B and

Beclin-1 expression whereas, decreased p62 expression. These results suggested that RAMS11 silencing led to autophagy induction and formation of autophagic flux. Our findings also implied that RAMS11 suppressed autophagy in CRC cell lines.

Most chemotherapy drugs promote programmed cell death process called apoptosis, however chemo-resistant cells does not respond to the therapy (Ricci and Zong, 2006). Apoptosis pathway is maintained by pro-apoptotic and pro-survival proteins that establish balance between cell survival and death by regulating Bcl-2 family proteins (Singh et al., 2019). The mitochondrial containment of Bcl-2 participate in intrinsic apoptosis by restricting oligomerization of BAX or BAK responsible for extended cell cycle (Singh et al., 2019). Bcl-2, Bcl-xL overexpression reduce apoptosis and facilitate immortalization of damaged cells, resulting in excessive proliferation and tumour development (Zhang et al., 2018, Singh et al., 2019). In addition, a protein complex of cytochrome C, APAF1, and dATP form apoptosome in cytosol, which initially activate caspase 9 and followed by activation of caspase 3, 6, and 7 to stimulate cellular phagocytosis process (Li and Yuan, 2008, Kim et al., 2015a, Singh et al., 2019). Our results suggested that the upregulation of RAMS11 promoted Bcl-2, Bcl-xL, and pro-caspase-9, whereas RAMS11 downregulation showed reduced expression of those proteins hence increased apoptosis. Connecting our findings on cell proliferation, autophagy, and apoptosis, we suggest that RAMS11 support cell proliferation in CRC by downregulating autophagy and apoptosis.

The AKT/AMPK/mTOR signaling is the major regulatory pathways associated with cellular autophagy, apoptosis, cell proliferation, migration, and angiogenesis in cancer (Memmott and Dennis, 2009, Paquette et al., 2018). The serine-threonine protein kinase mTOR consist of two functionally distinct complexes called mTORC1 and mTORC2. In order to maintain cellular growth, proliferation, migration, apoptosis, and autophagy, the protein complexes mTORC1 and

mTORC2 are activated by various stimulus, such as nutrient deprivation, stress, growth factors, and potential regulatory signalings (e.g. PI3K, AKT, MAPK, and AMPK) (Pópulo et al., 2012). Studies have shown that mTORC1 activation inhibits autophagy induction whereas mTORC2 indirectly activates mTORC1 to suppress autophagy (Codogno and Meijer, 2005, Paquette et al., 2018). The PI3K pathway activates mTORC2 by phosphorylatings AKT resulting activation of AKT and mTORC1 (Paquette et al., 2018). Another key regulatory signaling in mTORC1 dependent autophagy is AMP-activated protein kinase (AMPK), which also is activated in nutrient deprivation and stress condition (Wang et al., 2012). Therefore, AMPK is considered to be an "energy controller" of eukaryotic cells. The phosphorylation of AMPK induce autophagy by restricting mTORC1 and activating several murine proteins Ser317, Ser777, and Ser555 in stress and energy starvation conditions (Codogno and Meijer, 2005, Wang et al., 2012, Paquette et al., 2018, Qi et al., 2018). In this study, our key pathway investigation revealed that Dsi-RAMS11 potentially induced autophagy and apoptosis by phosphorylation of AMPK and inhibition of AKT and mTOR. This findings accomplished that dysregulated RAMS11 promotes carcinogenesis of CRC cells via phosphorylating AKT/mTOR, dephosphorylating AMPK, inhibiting autophagy, and reducing apoptosis process.

The EMT process activation comprises of losing intracellular adhesion and polarity to increase migratory and invasive properties of cells (Roche, 2018). The EMT induction promoted epithelial marker E-cadherin whereas reduced mesenchymal maker proteins N-cadherin, Vimentin, and Fibronectin (Ribatti et al., 2020). In addition, the EMT process is regulated by a number of transcription factors such as Snail, Sox2, ZEB1, and TWIST (Gao et al., 2015, Ribatti et al., 2020). These transcription factors regulate EMT by direct or indirect regulation of EMT marker proteins (Gao et al., 2015, Ribatti et al., 2020). Snail activates EMT by reducing E-cadherin and claudins,

and increasing Vimentin and Fibronectin in cancer (Kaufhold and Bonavida, 2014). Another well-established stem cell marker Sox2 play crucial roles in initiation and progression of carcinogenesis (Han et al., 2012). The previous research demonstration revealed that Sox2 knockdown potentially induces mesenchymal to epithelial transition (MET) process in CRC cells along with E-cadherin and Vimentin via regulating Wnt pathway (Han et al., 2012). In the current study, our result indicated that RAMS11 potentially enhanced EMT process to promote carcinogenesis. The downregulation of RAMS11 reversed the EMT process by increasing epithelial marker E-cadherin, decreasing mesenchymal markers N-cadherin and Vimentin, and reducing transcription factors Sox2 and Snail expressions in HCT-116 and SW480 cells. This demonstration indicated that RAMS11 may promote CRC progression and development of metastasis by achieving EMT regulated invasive and migratory characteristics of CRC cells.

To elaborate, EMT is a transiently occurring cellular reversible process where epithelial cells loses their epithelial characteristics and embrace spindle shaped, mesenchymal cellular morphology. However, mesenchymal cells more often reverse the process called MET (Dongre and Weinberg, 2019). Based on the phenotype of output cells, EMT is subcategorized into three classes. In type 1 EMT, primitive epithelial cells turned to motile mesenchymal cells which may undergo reversible MET process to form secondary epithelial cells. Type 2 EMT take places when secondary epithelial or endothelial cells accumulate to the interstitial spaces and turned to fibroblast tissue. Whereas, type 3 EMT is a branch of metastasis whereby epithelial cells depart from its primary site and migrate to the other distant tissue site (Zeisberg and Neilson, 2009). Based on the functional perspective, EMT is divided into complete EMT and partial EMT or hybrid EMT. In the complete EMT process, the epithelial cells entirely converted into mesenchymal phenotypes whereas in partial EMT cells exhibits both epithelial and mesenchymal characteristics

(Saitoh, 2018). It is believed that hybrid phenotypes exhibits aggressive invasive nature by propagating circulating tumour cells and cancer stem cells, and enhance resistance to the cancer therapies (Saitoh, 2018). Moreover, the cell with hybrid EMT act as cancer stem cells in most of cancer types. These cells possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer samples.

5.5 Conclusion

In summary, our study described the oncogenic roles of RAMS11 in CRC. We also demonstrated that downregulation of RAMS11 may provide a new branch of targeted therapy and better understanding of carcinogenesis via mTOR dependent activation of autophagy, promotion of apoptosis, and inhibition of EMT process. However, some limitations of this study include the absence of *in vivo* studies and verification using patient samples which might allow us to make stronger conclusions of our findings and to support RAMS11 as a potential cancer biomarker for CRC.

Chapter 6

Proteomic profiling of metabolic proteins as potential radiation therapy-biomarkers for colorectal cancer tumours

6.1 Introduction

Radiation therapy (RT) is one of the most common treatment options for most of cancer types (Berkey, 2010, Wisdom et al., 2019). Nearly two thirds of all cancers are treated with RT. The application of RT effectively inhibits growth and proliferation of cancerous cells by damaging DNA and eventually kill the cells (Jackson and Bartek, 2009). The major limitation for RT therapy is the damaging of normal cells within the irradiation area. However, normal cells have higher ability to repair themselves for retaining normal functions but cancerous cells lack efficient repairing ability (Begg et al., 2011).

In Chapter 1.4.1.2, we have reviewed the use of RT for CRC treatment. Clinically, RT is a crucial treatment modality, commonly recommended to treat rectal and sigmoid CRC due their anatomical compatibility (Agranovich and Berthelet, 2000). The early stages of CRC patients with localized tumours are usually treated with RT along with chemotherapy or surgical excision (Glynne-Jones and Kronfli, 2011). RT significantly boost up the treatment outcomes of surgery or chemotherapy by extending the overall survival and diseases free survival of the patients (Hatcher and Kumar, 2014). In addition, palliative RT is also effective in reducing the symptoms and extend survival of metastatic patients (Barrett et al., 2009). Equally, advanced techniques of RT for Stage-IV inoperable and elderly patients have been developed over the years, which enhanced the prognosis and disease control (Tam and Wu, 2019).

Tumour response to RT largely depends on the heterogeneity of the patients even if they are diagnosed with the similar cancer type (Story and Wang, 2018). In the past few years, improvements have been made in RT techniques associated with precise delivery of radiation to localized tumours (Meehan et al., 2020). However, there are still some obstacles for establishing personalized RT due to heterogeneity in the location, physiology, and genomic features of the tumours (Bratman et al., 2017). Particularly, it is nearly impossible to establish RT competent to the molecular biology of diverse cancer types (Bratman et al., 2017, Story and Wang, 2018, Meehan et al., 2020). In order to improve the personalized RT in cancer management, we propose that prognostic biomarkers can provide predetermining information about intrinsic radio-sensitivity of tumours or response to RT. Undoubtedly, it can help clinicians to determine individual treatment options and reduce RT toxicity to reach maximum therapeutic outcome (Agranovich and Berthelet, 2000, Meehan et al., 2020). Hence, prognostic and predictive biomarkers are fundamental to achieve better treatment outcomes in precision RT.

The tumour radiophenotype is regulated by a number of factors including clonogene number, rate of DNA damage, ratio of cell growth, immunogenicity of cells, and oxygenation of cells (Bratman et al., 2017). Precise genomic biomarkers for RT could reflect an universal radiophenotype for a distinct tumour type yet long-term prospective study is need to establish it (Eschrich et al., 2009). Over the past decade, gene mutation based prognostic biomarkers have been proposed for systemic single-agent-targeted treatment approach. For example, KRAS/BRAF/NRAS are the most commonly used mutation based biomarkers for metastatic CRC or metastatic melanoma (Sethi et al., 2013, Ree and Redalen, 2015, Carlomagno et al., 2017). Apart from this, cancer antigen 125 (CA125), carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), prostate-specific antigen (PSA) are currently used clinical biomarkers for OVC, PNC, CRC and PRC but

they are not RT specific (Kulasingam and Diamandis, 2008, Hanash et al., 2011). In recent years, the rapid development in high-throughput omics technologies has identified numerous biomarkers candidate for cancer diagnosis, prognosis, and treatment. In CRC, the KRAS and NRAS mutations are the most conventionally used prognostic biomarkers for anti-EGFR therapy, and chemotherapy in metastatic CRC patients (Adeola et al., 2014, Blons et al., 2014, Deng et al., 2015, Chuang et al., 2020). None of these studies has concluded the correlation of their molecules with RT response. Therefore, in this study, we focus on the radio-responsiveness of CRC tumours and aim to identify prognostic biomarkers to predict RT treatment outcome.

6.2 Methods

In collaboration with Dr. TAM Shing Yau, Department of Health Technology and Informatics, The Hong Kong Polytechnic University, we generated CRC xenograph tumours in nude mice (Chapter 3.2.2, and Chapter 3.2.3) and ranked the tumours according to their shrinkage in size as a measure of radio-responsiveness. 3 good responders and 3 poor responders, and 3 unirradiated controls were included for proteomics analysis (Chapter 3.3.1 to Chapter 3.3.4).

6.3 Results

6.3.1 Overview of proteomics data

In order to analyse the protein expression changes among good responders, poor responders, and unirradiated control tumours, we employed high throughput mass spectrometry (MS) proteomics analysis. MS analysis identified a total of 1416 proteins. Among the identified proteins, 106 proteins were differentially expressed (DEPs) between control and poor responders (fold change ≥ 2, $p \leq 0.05$) (Figure 6.1.A) where 39 and 67 proteins were up and downregulated respectively (Figure 6.1.A). On the other hand, 570 proteins were DEPs between control and good responders

(fold change ≥2, $p \leq 0.05$) (Figure 6.1.B) of which 253 and 317 proteins were up and downregulated respectively (Figure 6.1.B). The overall expression pattern among groups were displayed in heat map (Figure 6.1.C) and the heat map data showed that the expression pattern among replicates are consistent (Figure 6.1.C).

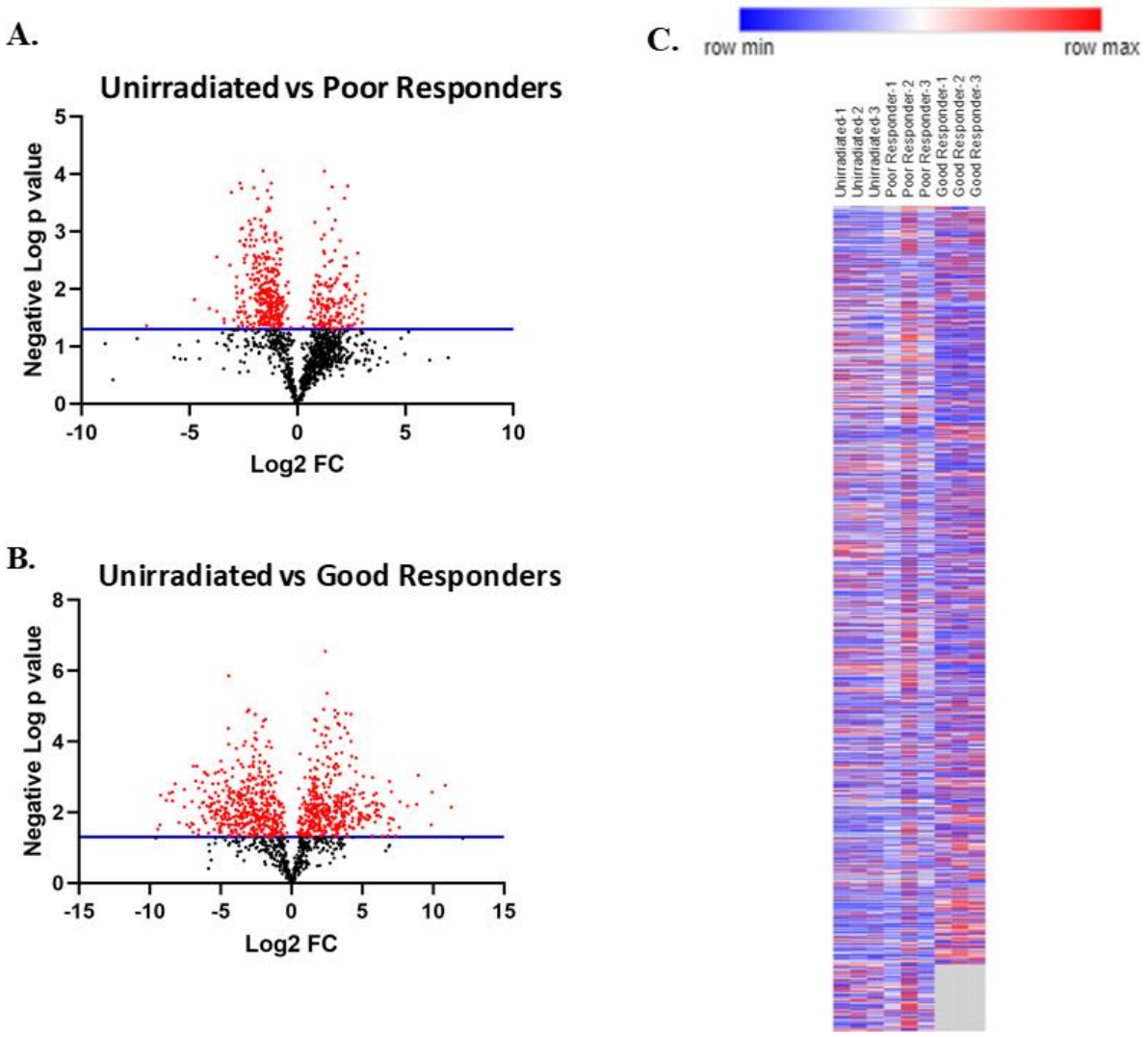


Figure 6.1. Overview of proteomics data. (A) Volcano plot analysis of differentially expressed proteins (DEPs) between unirradiated vs poor responders, (B) Volcano plot analysis of differential expressed proteins between unirradiated vs good responders. Colored points represent differentially expressed proteins (p value ≤ 0.05, fold change ≥ 2.0). (C) Heat-map analysis of differential expressed proteins among groups. Each row corresponds to one gene, green and red indicate down- and upregulation of respective proteins.

6.3.2 Gene ontology (GO) and pathway analysis of significantly altered proteins

To determine the multiple biological functions, we employed Enrichr, a functional enrichment analysis database, to classify the genes according to their respective gene ontology (GO) terms. GO biological process function of Enrichr showed significantly altered proteins in both good and poor responders group enriched for neutrophil degranulation and neutrophil related immunity with regard to control (Figure 6.2.A, D). At the molecular function level, secretory granule lumen processes are enriched in poor responders group, whereas focal adhesions term was in the top GO molecular function category in good responders group (Figure 6.2.B, E). GO interpretation of cellular component revealed that, most of the altered proteins are associated with RNA binding function in both group (Figure 6.2.C, F). The GO term for each group are associated with similar term but the degree of association is different.

We further examined the association of these altered proteins with pathways by Enrichr and the results showed that, KEGG proteasome are enriched in poor responders followed by spliceosome pathways (Figure 6.3.A) but in case of good responders, KEGG ribosome pathway and glycolysis (Figure 6.3.B) are enriched, suggesting that a number of radio-resistance pathways are activated in case of poor RT responders.

Next, we identified hallmark pathways associated with altered proteins in good responders group compare to control group by Gene Set Enrichment analysis (GSEA) and the data revealed that, epithelial to mesenchymal transition (EMT) was de-regulated (Figure 6.3.C). Among the de-regulated genes associated with EMT, CALU, CAPG, GAGLN, PPIB, TPM2, and VIM are found to be downregulated in good responders but not the poor responders and the control (Figure 6.3.D).

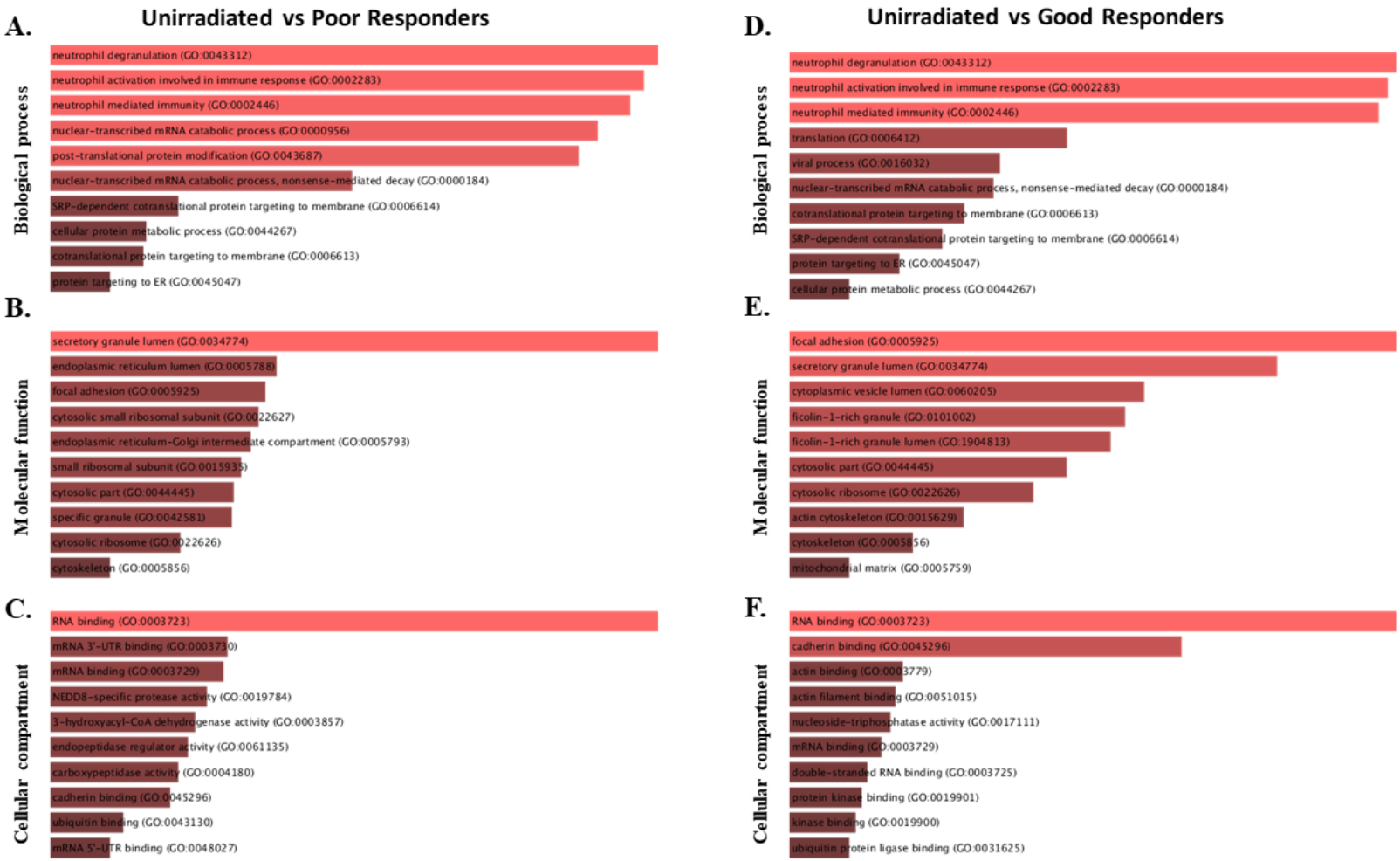


Figure 6.2. Gene ontology (GO) analysis of significant differential expressed proteins (DEPs). (A-C) Bar chart showed the top 10 GO terms for biological process, molecular function and cellular component respectively between unirradiated vs poor responders with significant DEPs. (D-F) Bar chart showed the top 10 GO terms for biological process, molecular function and cellular component respectively between unirradiated vs good responders with significant DEPs. Bar charts length and color represents the significance of respective term.

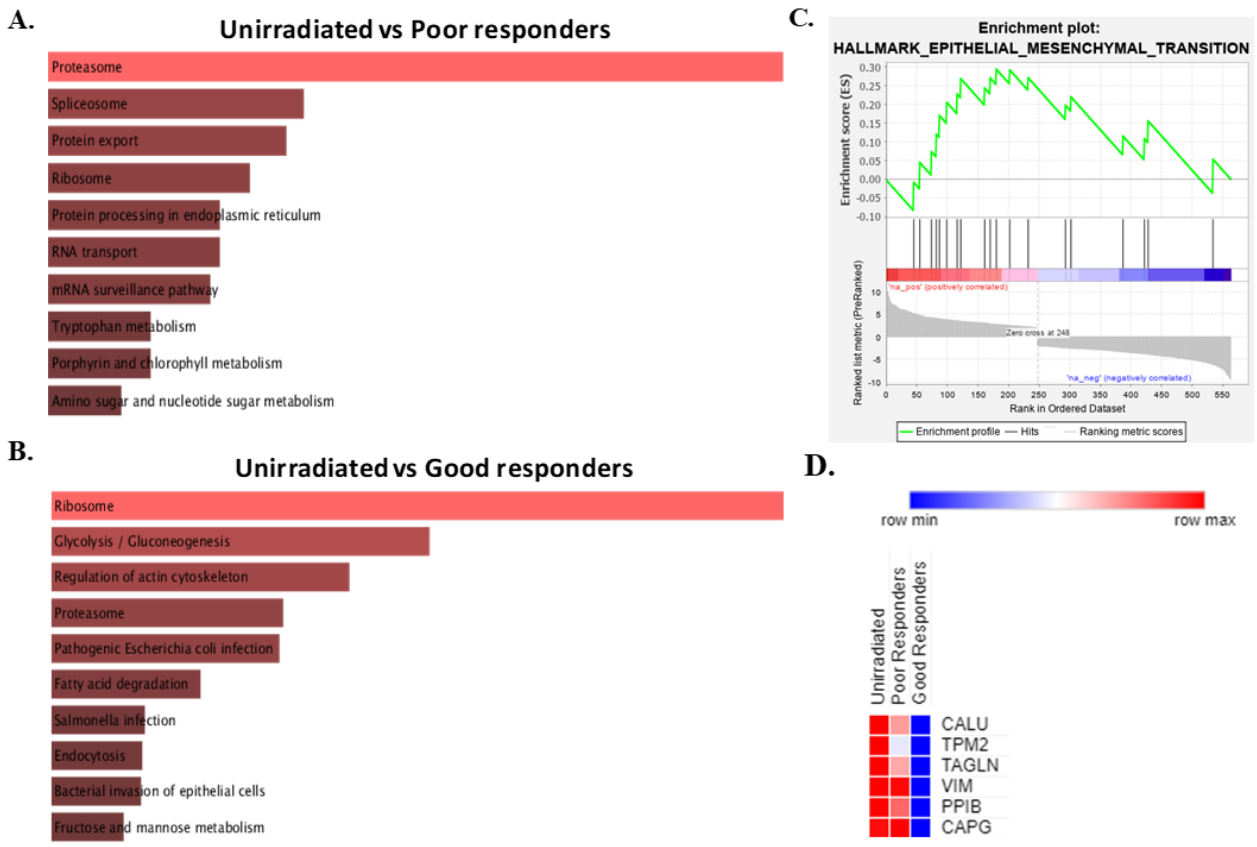


Figure 6.3. Pathway and gene set enrichment analysis (GSEA) of significant differential expressed proteins. (A) KEGG pathway analysis between unirradiated vs poor responders group with significant DEPs. (B) KEGG pathway analysis between unirradiated vs good responders group with significant DEPs. Bar charts length and darkness represents the significance of the respective terms. (C) GSEA of significant differential expressed proteins data with hallmark gene sets between unirradiated vs good responders group. Data related to EMT are shown here. The green curve represents the enrichment score. (D) Heat map analysis of key EMT proteins among groups. The expressions of these 6 genes in the good responders group are clearly different from the poor responders and control groups. Each row represents one protein, blue and red indicate down- and upregulation of respective proteins.

6.3.3 High expression of phosphoglycerate kinase 1 (PGK1) and other metabolic proteins might serve as a potential biomarkers for radio-resistance in CRC

To find out the expression differences between poor and good responders, we employed WEB-based Gene SeT AnaLysis Toolkit (WebGestalt), a functional enrichment analysis web tool to generate rank-based pathways based on expression changes among groups. Based on WebGestalt results, a number of pathways are dysregulated between poor and good responders (according to Wikipathway) (Figure 6.4.A). Interestingly, the metabolic reprogramming of CRC was highly depreciated in the good responders group. Particularly, the proteins involved in this particular pathway were downregulated in good responders compared to poor responders (Figure 6.4.A, B) but the protein expression patterns were almost similar between control and bad response group (Figure 6.4B). Literature search of metabolic reprogramming of CRC associated proteins in our dataset showed that they are all associated with CRC pathogenesis and upregulation of these proteins are linked to worse CRC diagnosis/ prognosis (Table 6.1). Moreover, STRING based protein-protein interactions data revealed that, these proteins are highly inter-connected with each other (Figure 6.4.C).

Next, we want to know whether any of these proteins has prognostic value for CRC. To do this, we performed extensive literature search and found proteins in our list has been well studied in CRC.

To validate the proteomics data, we performed WBt analysis on the tumours and confirmed that each tumour in the good responders group showed lower protein expression that the poor responders and the controls (Figure 6.4.D).

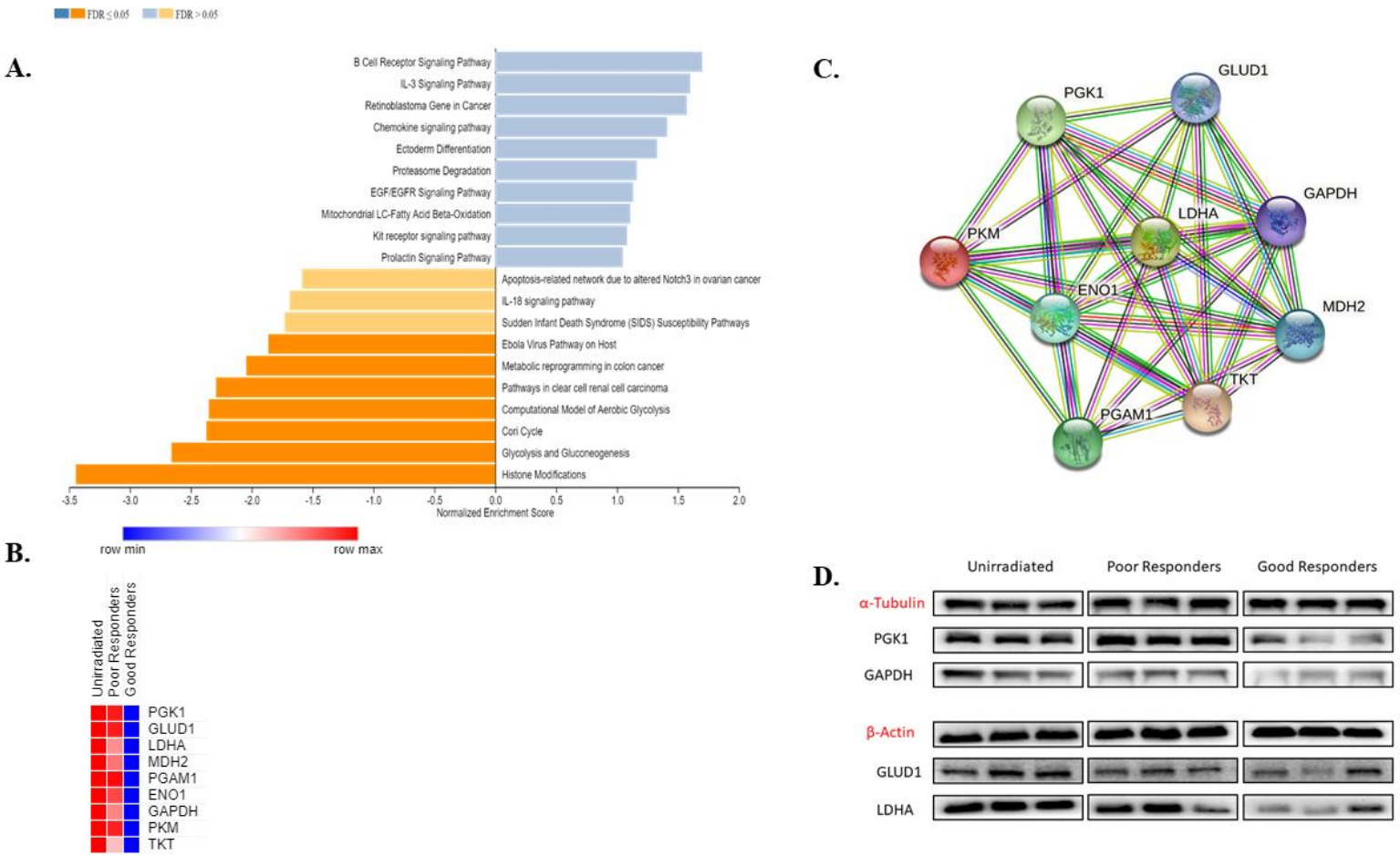
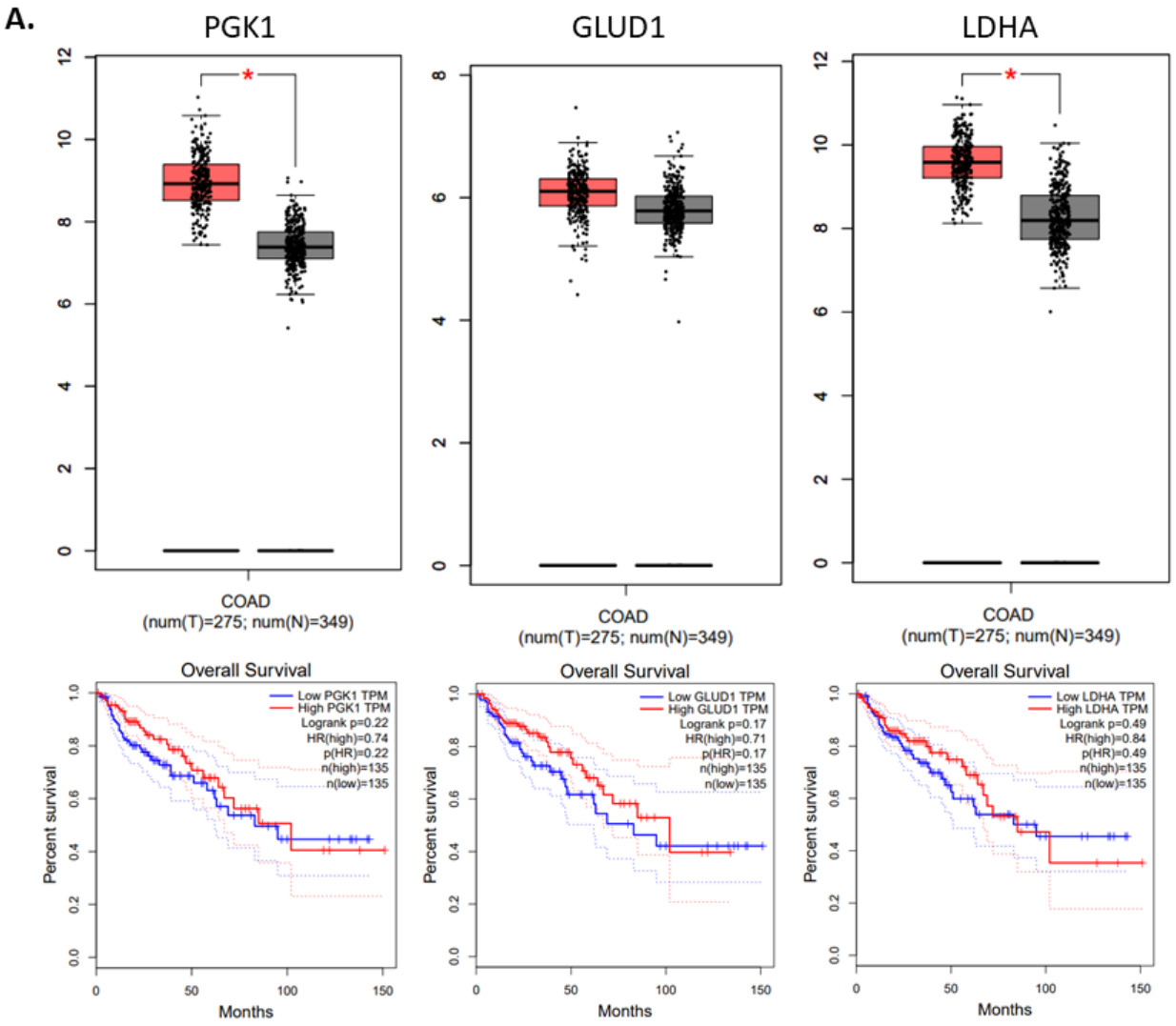
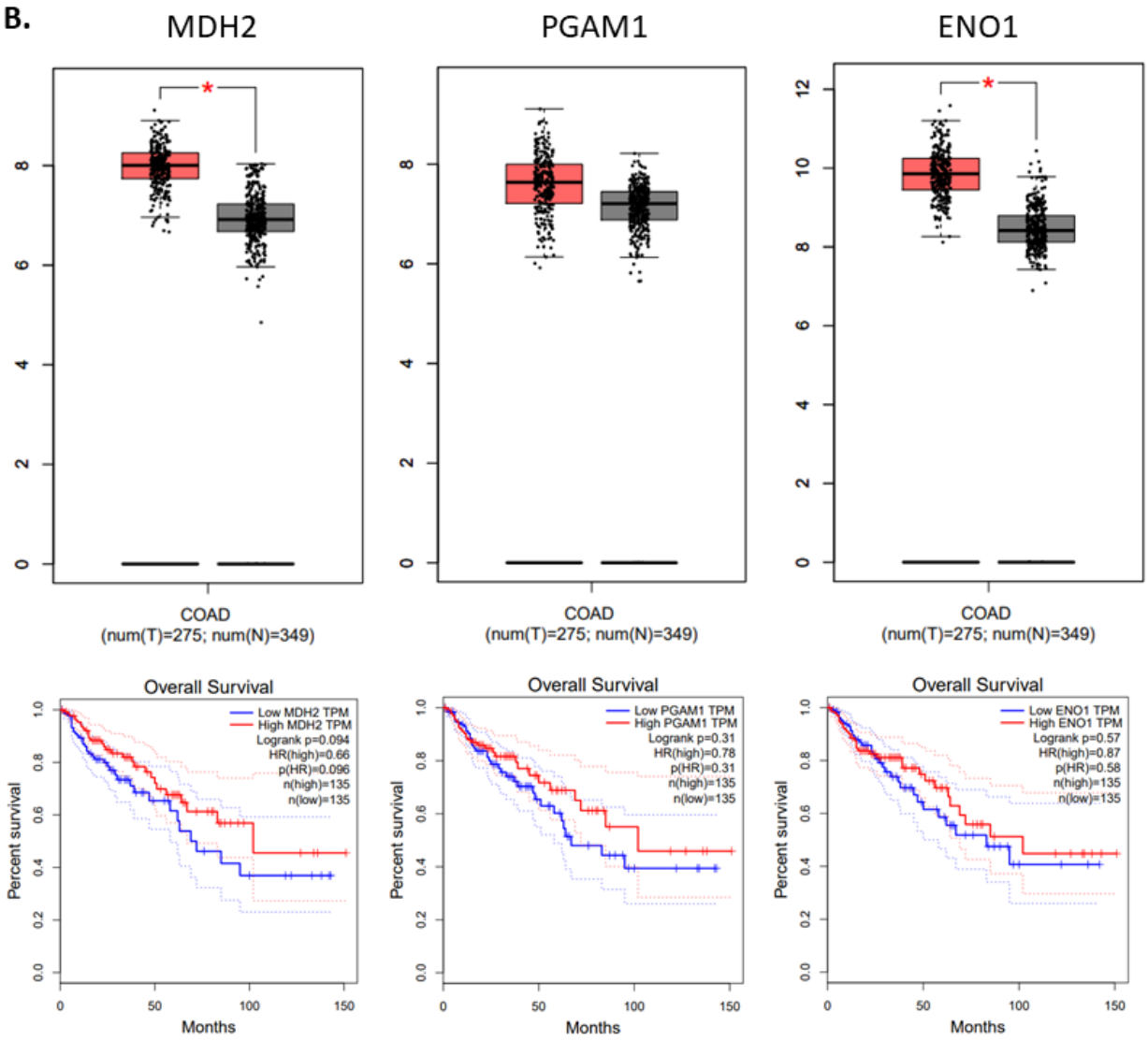


Figure 6.4. Pathway and functional analysis of significant differential expressed proteins between good and poor responders group. (A) Wikipathway analysis of between good vs poor responders group with significant DEPs. Pathways having false discovery rate (FDR) smaller than or equal to 0.05 are darker shades and while light shades are the opposite. (B) Heat map analysis of metabolic regulation of CRC proteins among groups. Each row represents one protein, green and red indicate down- and upregulation of respective proteins. (C) Protein-protein interaction (PPI) network generated by STRING software of proteins related to metabolic regulation of CRC. (D) WBt analysis of PGK1, GAPDH, GLUD1, and LDHA between unirradiated, poor responders and good responders group.

6.3.4 Cross-validation of biomarkers with TCGA dataset

RT effectively destroys tumour cells by damaging various genetic contents such as DNA, RNA, proteins, and enzymes (Kaliberov and Buchsbaum, 2012). As proteins play central roles in biological functions and molecular mechanisms in normal and disease state, we further validated the gene expression of our RT prognostic marker proteins with publicly available dataset TCGA-COAD and web-based platform the Gene Expression Profiling Interactive Analysis (GEPIA). The TCGA gene expression analysis showed that *PGK1, LDHA, MDH2, ENO1, GAPDH, PKM,* and *TKT* expressions were significantly upregulated in CRC tissues compared to adjacent normal samples (Figure 6.5.A-C). The expressions of *GLUD1*, and *PGAM1* were not significantly upregulated but they followed the same upregulation trends with other proteins (Figure 5.A-C). We also explored the association of these proteins with patient survival. The curve was plotted using expression levels using TCGA-COAD dataset. We found that the upregulation of genes are associated with reduced overall survival (Figure 6.5.A-C).


A.
PGK1
GLUD1
LDHA
COAD
(num(T)=275; num(N)=349)
Overall Survival
Low PGK1 TPM
High PGK1 TPM
Logrank p=0.22
HR(high)=0.74
p(HR)=0.22
n(high)=135
n(low)=135
Low GLUD1 TPM
High GLUD1 TPM
Logrank p=0.17
HR(high)=0.71
p(HR)=0.17
Low LDHA TPM
High LDHA TPM
Logrank p=0.49
HR(high)=0.84
p(HR)=0.49
Percent survival
Months


B.
MDH2
PGAM1
ENO1
COAD
(num(T)=275; num(N)=349)
Overall Survival
Low MDH2 TPM
High MDH2 TPM
Logrank p=0.094
HR(high)=0.66
p(HR)=0.096
n(high)=135
n(low)=135
Low PGAM1 TPM
High PGAM1 TPM
Logrank p=0.31
HR(high)=0.78
p(HR)=0.31
n(high)=135
n(low)=135
Low ENO1 TPM
High ENO1 TPM
Logrank p=0.57
HR(high)=0.87
p(HR)=0.58
n(high)=135
n(low)=135
Percent survival
Months

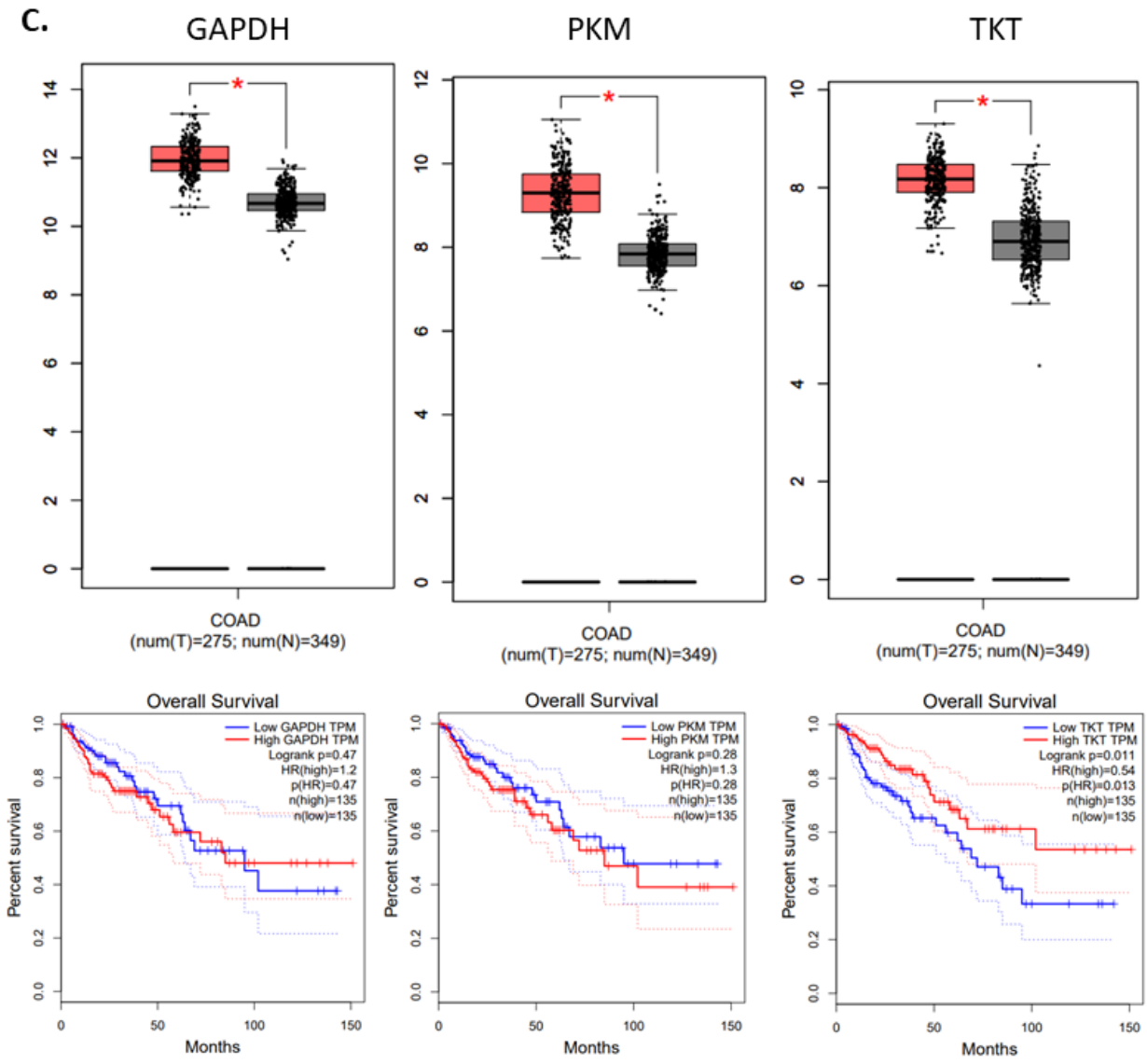


Figure 6.5. Box-plot of key metabolic proteins associated with RT responsiveness. The expressions of metabolic proteins were extracted from TCGA-COAD dataset. Number of tumour samples-275, number of normal tissues-349, *$P < 0.01$. TPM (Transcript per million), HR (Hazard ratio).

6.4 Discussion

There are different treatment modalities such as surgery, RT, and chemotherapy for managing CRC and the treatment selection mainly depends on the cancer staging. Although surgery is the first choice for CRC management, the roles of RT in CRC should not be undermined. RT is usually applied to many advanced stages CRC cases along with chemotherapy or surgery (Lupattelli et al., 2017, Chen et al., 2019a). Currently, 3-dimensional conformal RT (3DCRT) is the standard RT approach as 3D planning allows dose analysis of treatment volumes by dose volume histogram (Hathout et al., 2017). Dose to normal tissues could be well-controlled to reduce RT-mediated secondary symptoms and improved the RT effectiveness. To reduce the RT-associated complications, many literatures suggested the changing of the dose schemes or target volume for individualized treatment for improving the treatment outcomes (Krengli et al., 2010, Yaromina et al., 2012, Forker et al., 2015). In this project, we aim to identify biomarkers that may be used to predict radio-responsiveness of CRC. By using mouse xenograft model, a list of candidate proteins were identified.

Table 6.1. Roles of shortlisted metabolic proteins in CRC

Gene symbol	Name	Reported functions in CRC	References
PGK1	Phosphoglycerate kinase 1	PGK1 secreted from CRC tumours promotes cell proliferation, angiogenesis, metastasis, and facilitate 5-FU resistance	(Shichijo et al., 2004, Ahmad et al., 2013)
GLUD1	Glutamate dehydrogenase 1	GLUD1 regulates cellular energy generation under hypoxic condition, act as a prognostic and metastatic biomarker in CRC.	(Liu et al., 2015, Miyo et al., 2016)
LDHA	Lactate dehydrogenase A	Critical enzyme LDHA promotes production of ATP by regulating glycolysis in both normal and hypoxic conditions. With significant correlation of LDHA with HIF1α, HIF2α, GLUT-1, VEGFA, and VEGFR1, LDHA is proposed to be a potential prognostic biomarker in CRC.	(Azuma et al., 2007, Untereiner et al., 2017, Mizuno et al., 2020)
MDH2	Malate dehydrogenase 2	MDH2 is one of the key regulatory enzymes of tricarboxylic acid (TCA) cycle of NAD/NADH coenzyme system.	(Goward and Nicholls, 1994, Naik et al., 2014, Ban et al., 2016)

		MDH2 inhibitor could be a potential anticancer target in CRC management.	
PGAM1	Phosphoglycerate Mutase 1	The upregulation of PGAM1 associated with glycolysis process and dysregulation of PGAM1 promotes metastatic process of CRC.	(Lei et al., 2011, Fedorova et al., 2019)
ENO1	Enolase 1	ENO1 involves in glycolysis and promotes CRC growth, migration, and metastasis through AMPK/mTOR pathway.	(Zhan et al., 2017, Cheng et al., 2019, Fedorova et al., 2019)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	The dysregulation of GAPDH is commonly associated with various carcinogenesis including CRC where Vitamin C selectively destroys mutated CRC cells (KRAS and BRAF) through targeting GAPDH.	(Tarze et al., 2007, Guo et al., 2013, Yun et al., 2015)
PKM	Pyruvate kinase M1/2	PKM catalyses in glycolysis process and glucose consumption whereas, PKM promotes cell growth and migration in CRC cells.	(Yang et al., 2014, Kuranaga et al., 2018)
TKT	Transketolase	TKT is one of glycolytic and pentose-phosphate pathway enzymes associated with CRC metabolic reprogramming.	(Chen et al., 1999, Vizán et al., 2009)

A number of imaging approaches have been developed to quantify the RT response to the tumours. This approaches mainly measure the degree and rate of tumour shrinkage against the RT (Woliner-van der Weg et al., 2018, Meehan et al., 2020). Particularly, the Response Evaluation Criteria in Solid Tumour (RECIST) theory has been well established and commonly applied in clinical settings. RECIST theory subdivides the tumours into groups of complete disappearance, partial disappearance, unchanged or progressive disappearance. The imaging techniques such as, X-ray, computed tomography (CT), magnetic resonance imaging (MRI), and FDG positron emission tomography (FDG-PET) may be employed to determine the shrinkage of tumour after application of RT (Eisenhauer et al., 2009, Puaux et al., 2011, Gruber et al., 2013). However, these techniques only measure the rate and extent of tumour size reduction, the underlying molecular biology is much less studied. In addition, tumours that were nonresponsive to RT were only distinguished from responsive tumours after the end of the whole treatment period. Therefore, nonresponsive tumours continued its progression, the delayed in selecting alternative treatment strategies resulted in reduced overall survival (Cheung, 2006, Baskar et al., 2012, Meehan et al., 2020).

Proteins are regulatory components of human body and it is believed that cancer is the result of abnormalities in genetic alterations or environmental stress (Donnelly and Storchová, 2014). Another potential carcinogenic mechanism is the alteration of proteomics balance of the body due to formation of defective proteins, mutations of specific proteins, and deletion or degradation of regulatory proteins responsible for tumour suppressor activity (Jain, 2002, Mosca et al., 2013, Sallam, 2015). It has been reported that genes and proteins are key regulatory components in cell death process induced by RT (Wahba et al., 2016, Wang et al., 2018b). Similarly, our study proposed that the RT responsiveness is associated with a group of differentially expressed proteins

in CRC tumours. Our GO study revealed that RT has altered many biological functions of CRC including, metabolism, neutrophil degranulation, focal adhesion, RNA binding functions, and EMT.

The poor prognosis for most cancers is due to the development of metastasis where EMT enhances the cellular migration properties (Brabletz et al., 2018, Wang et al., 2020). EMT reduces tight junction between cells, basal polarity, and cytoskeletal structure to induce cellular motility, resulting development of invasiveness or metastatic phenotype of CRC (De Bosscher et al., 2004, Vu and Datta, 2017). GSEA analysis demonstrated that the hallmark EMT proteins are significantly downregulated in the tumours of good RT response but upregulated in both unirradiated and poor responses tumours. Our list of EMT regulatory proteins included, CALU, CAPG, PPIB, TAGLN, TPM2, and VIM, they regulated the EMT process in CRC tumours. The recent findings indicated that CALU play key roles in CRC development and metastasis (Nasri Nasrabadi et al., 2020), TPM2 is implicated with potential roles in CRC development (Zhao et al., 2019). Similarly, transgelin (TAGLN) is found to be a key regulatory factor associated with later stages CRC where it promotes CRC carcinogenesis through TGFβ signaling (Elsafadi et al., 2020). Another EMT marker protein vimentin (VIM) expression was upregulated to induce EMT, resulting poor prognosis and reduced survival of CRC patients (Du et al., 2018). Moreover, the overexpression of capping actin protein (CAPG) contributes in CRC migration and peptidylprolyl isomerase B (PPIB) promotes chemoresistance in CRC by reducing p53 and binding with MDM2 (Choi et al., 2018, Tsai et al., 2018b). These literature supported our hypothesis that the specific EMT proteins may be used as prognostic markers for tumour radio-responsiveness.

In order to establish RT-prognostic biomarkers, web-based functional analysis was performed and we identified a number of pathways dysregulation between poor responding and good responding

groups. Interestingly, the metabolic reprogramming of CRC is highly depreciated in good RT responses group compared to unirradiated control and poor RT responses groups. Importantly, the 9 shortlisted proteins namely PGK1, GLUD1, LDHA, MDH2, PGAM1, ENO1, GAPDH, PKM, and TKT are found to be upregulated in unirradiated control and poor responses groups but downregulated in good responses group. In addition, these proteins are highly connected with each other. Extensive literature studies revealed that they are highly associated with CRC carcinogenesis and poor prognosis.

We performed extensive literature studies on the shortlisted metabolic proteins and revealed that PGK1, the top proteins on our list, is the first ATP-generating enzyme in glycolysis, associated with CRC progression and development of metastasis (Ahmad et al., 2013). In addition, PGK1 glycosylation promotes CRC proliferation and growth by regulating glycolysis and TCA cycle (Nie et al., 2020). Similarly, GLUD1 is a crucial catalytic enzyme of glutamine metabolic pathway and related to poor prognosis of CRC. It is proposed that GLUD1 might be a novel prognostic marker for metastatic CRC and new therapeutic target in refractory CRC (Liu et al., 2015, Miyo et al., 2016). Equally, LDHA is another critical regulator of glycolysis and ATP production. It is upregulated in many cancer types. The association of LDHA with HIF-α, GLUT-1, NRP1, VEGFA, and VEGRF1 may serve as surrogate markers in CRC (Azuma et al., 2007, Untereiner et al., 2017, Mizuno et al., 2020). MDH2 is another regulatory enzyme of mitochondrial TCA cycle. It is shown that MDH2 inhibitor could be a potential therapeutic strategy of CRC (Goward and Nicholls, 1994, Naik et al., 2014, Ban et al., 2016). Research showed that PGAM1 upregulation promoted glycolysis and development of metastasis in CRC (Lei et al., 2011, Fedorova et al., 2019). Subsequently, ENO1 is another conserved glycolytic enzyme which is upregulated in many cancer types including CRC. The upregulation of ENO1 promotes CRC

growth, migration, and metastasis via regulating RAB1A/AMPK/mTOR signalings (Zhan et al., 2017, Cheng et al., 2019). The widely accepted housekeeping gene GAPDH is another essential glycolytic regulatory enzyme. It is commonly upregulated in 21 cancer classes, and is a key regulator of mitochondrial membrane permeabilization in pro-apoptic stages (Tarze et al., 2007, Tarrado-Castellarnau et al., 2017). Interestingly, vitamin C selectively destroy KRAS and BRAF mutated CRC cells by regulating GAPDH (Yun et al., 2015). The metabolic enzyme PKM was found to regulate metabolism process of CRC cells whereas the upregulated PKM enhances migration and adhesion of CRC cells by targeting STAT3 (Yang et al., 2014, Kuranaga et al., 2018). Apart from this, pentose phosphate pathway regulatory enzyme TKT can effectively distinguish the carcinoma and normal tissues in colorectal tissues (Chen et al., 1999, Vizán et al., 2009). We believe these proteins are upregulated in CRC tumours and effective RT reduced their expressions and tumour size. Thus, proteins may serve as potential RT biomarkers in CRC management.

This project has great potential to be extended to explore the roles of metabolic proteins in radio-responsiveness. Future project may explore the expression of specific metabolic proteins in biopsy samples collected before, after, and at mid-course of RT to predict tumour responsiveness. If poor response is predicted, the additional RT dose along with chemotherapy may be indicated by oncologist. However, because it is an intervention that may be traumatic to the patients and there may be complications associated with it. So more concrete scientific evidences are needed before proceeding into large-scale clinical trials.

6.5 Conclusion

Overall, using high through-put proteomics analysis, we isolated 9 metabolic proteins which regulate pathways such as glycolysis (PGK1, PGAM1, ENO1, PKM, TKT), ammonia detoxification (GLUD1), carcinogenesis (LDHA, GAPDH), and drug responses (MDH2). The expressions of PGK1, GLUD1, LDHA and GAPDH were validated in the tumours by WB. Our results suggested that these proteins may be used as potential biomarkers for RT response in CRC.

Chapter 7

Overall discussion and conclusion

In this thesis, our first aim was to determine the potential roles of autophagy-modulating lncRNAs as novel CRC biomarkers *in vitro* and the second aim was to evaluate the metabolic proteins to establish RT-predictive biomarkers in CRC tumours *in vivo*. To achieve the first goal, roles of two autophagy-modulating lncRNAs CASC9 and RAMS11 was explored in CRC carcinogenesis (Chapter 5 and Chapter 5). In our investigation, we performed knockdown of CASC9 and RAMS11 in order to identify their oncogenic roles and molecular mechanisms in CRC carcinogenesis. For the second objective, we used tumours generated from a mouse xenograft model and high-throughput proteomics analysis to identify a group of metabolic protein that are associated with good RT responses *in vivo* (Chapter 6). Our key findings revealed that these metabolic proteins may be used as predictive markers for RT responsiveness in CRC tumours via regulating EMT pathway.

7.1 Autophagy-modulating lncRNAs are potential targets for CRC diagnosis and prognosis

Accumulating evidences suggested that lncRNAs sometimes behaved like regulatory molecules and are widely involved in the signaling pathways responsible for cell growth, development, and metabolic processes. In CRC, lncRNAs are associated with each stage of tumour initiation, progression, and metastasis by regulating major oncogenic signaling cascades. In our preliminary study, we prepared untreated, rapamycin-treated, and chloroquine-treated HT-29 cell for NGS. These samples represent control, autophagy-stimulated, and autophagy-inhibited CRC cells. After RNA sequencing, we identified 32 differentially expressed lncRNAs between autophagy-

stimulated and control samples. CASC9 was selected because CASC9 was the most differentially expressed lncRNA in the preliminary experiment.

In Chapter 4, we explored the biological and physiological roles CASC9 in CRC along with its molecular mechanisms. In the recent years, the studies of CASC9 drawn more and more attention due to their abnormal expression and transcript variants associated with the pathogenesis of many cancer types. Our study revealed that CASC9 is highly upregulated in CRC tumours and cell lines compared to normal tissues and cells. The upregulation of CASC9 in CRC associated with the reduced overall survival of patients. To evaluate the biological and physiological function, gene silencing technique was performed with CASC9 in CRC cells. The results indicated that silencing of CASC9 reduced cell proliferation, growth, and migration in vitro. The molecular mechanisms revealed that silencing of CASC9 promoted autophagy via phosphorylating AMPK, reducing AKT/mTOR, and altering EMT process.

Following the techniques described in Chapter 4, we explored the roles of a newly identified lncRNAs RAMS11. The previous study reported that RAMS11 overexpressed in metastatic CRC and it promotes invasive phenotypes in vitro and in vivo. Our study explored the molecular mechanisms of RAMS11 in CRC cells associated with aggressive phenotype. The mechanistic evaluation revealed that downregulation of RAMS11 significantly induce autophagy, promote apoptosis, downregulate AKT and mTOR signaling via AMPK pathway, and inhibit EMT process (Chapter 5). Together with, our results revealed that CASC9 and RAMS11 promoted CRC via inhibiting autophagy and apoptosis, phosphorylating AKT and mTOR via AMPK pathway, and promoting EMT. Figure 7.1 summarized the results of Chapter 4 and 5.

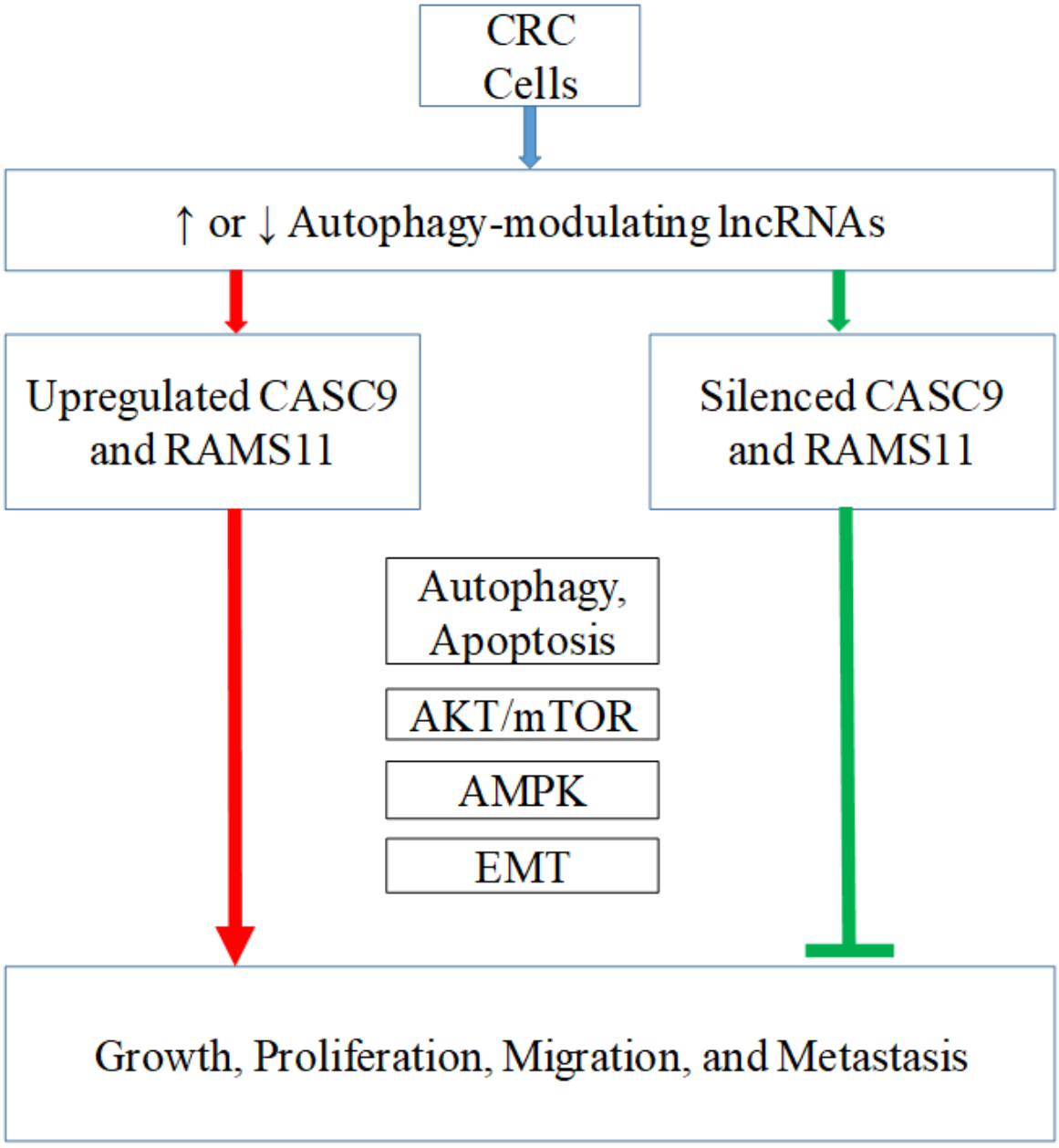


Figure 7.1. Autophagy-modulating lncRNAs are potential targets of CRC

So far, many molecular mechanism have been reported to be responsible for the initiation and progression of CRC. We focused on autophagy, AKT, AMPK, EMT, and mTOR because they are important regulatory pathways (Memmott and Dennis, 2009, Paquette et al., 2018, Koveitypour et al., 2019). The mutations in any of these regulatory pathways lead to the development of CRC (Koveitypour et al., 2019) whereas targeting them could be a novel approach in the management of CRC. Autophagy is one of most important mechanism associated with tumour progression and inhibition. However, the exact molecular mechanisms of autophagy in carcinogenesis not clearly defied yet due to their dual sword activity (Levine, 2007, Grácio et al., 2017). Besides, it is well established that lncRNAs promote or reduce carcinogenesis via mTOR pathway and regulating a cascade or autophagy-related genes (Yang et al., 2017, Peng et al., 2020, Zhang et al., 2020a). Among various components associated with the autophagy pathway, mTOR is a major element. It is a member of serine-threonine protein kinase PI3K-related kinase family. PI3K activate mTOR and lead to indirect inhibition of autophagy through phosphorylation of AKT (Dunlop and Tee, 2014).

The central regulator of eukaryotic cells metabolism is AMPK which activated during cellular ATP deficiency. AMPK plays critical roles in cell growth, metabolism, autophagy and cell polarity during deprivation of nutrients and growth factors (Mihaylova and Shaw, 2011). In autophagy, AMPK activates ULK1 (autophagy initiating kinase) and phosphorylate TSC2 to suppress mTORC1 activity leading to autophagy induction (Jang et al., 2018).

The poor prognosis for most cancer is due to development of metastasis where EMT comprise of losing intracellular adhesion and polarity to eventuate migratory and invasive properties of cells resulting in development of metastasis (Brabletz et al., 2018, Wang et al., 2020). The EMT induction promoted epithelial marker E-cadherin whereas, reduced mesenchymal maker proteins

N-cadherin, Vimentin, and Fibronectin (Ribatti et al., 2020). Subsequently, a number of transcription factors such as Snail, Sox2, ZEB1, and TWIST regulate EMT pathways via direct or indirect regulation of EMT proteins (Gao et al., 2015, Ribatti et al., 2020). Our results indicated that CASC9 and RAMS11 promote CRC metastasis through promoting EMT. In conclusion, our results suggested that CASC9 and RAMS11 are important CRC regulatory lncRNA and they may be used as potential biomarkers in CRC.

The application of the lncRNAs cancer biomarkers can be 2 folds. First, if we can detect the level of CASC9 and RAMS11 in tumour biopsy by colonoscopy during the diagnosis of cancer. The lncRNAs profile may support the aggressive behaviour of the tumour and the clinicians may prescribe more aggressive treatment for the patient. Moreover, to establish lncRNAs based biomarkers, it is necessary to analyse patient's databases or collect new patient's samples, and then draw association with the clinical data. However, so far, no studies have been performed on blood or other body fluid samples regarding CASC9 and RAMS11 in CRC yet. Secondly, the knowledge gained from the study of CASC9 and RAMS11 revealed the important molecular pathways involved in CRC carcinogenesis. We cannot directly knockdown these lncRNAs systemically, but new drugs may target to the molecular pathways involved. For example, there has been exploration of the use of mTOR inhibitor or rapamycin to stimulate autophagy in cancer patients. However, the outcomes of this therapy is not at satisfactory level yet, further developments may need to overcome it.

7.2 Metabolic proteins as potential RT biomarkers for CRC tumours

When discussing about treatment options for CRC, many people would think of surgical removal of the tumours but neglected the role of RT. This miss concept may be because the intestines are mobile and it is impossible to irradiate the mobile tumours. In fact, most of the CRC are found in

the sigmoid colon and rectum. For the inoperable tumours in this region, chemoradiation therapy is very common. The literature studies indicated that RT can be applied in many advanced stages CRC along with chemotherapy or surgery (Lupattelli et al., 2017, Chen et al., 2019a). However, the effectiveness of RT reduced drastically due to development of RT-associated secondary complications. To reduce the RT-associated complications, many literatures suggested that changing the dose schemes or volume to individualize can improve the treatment outcomes (Krengli et al., 2010, Yaromina et al., 2012, Forker et al., 2015). Importantly, it has been established that predictive markers can improve the individualized RT outcomes however, there is no reliable prognostic markers for RT responsiveness at all.

In Chapter 6, we used the CRC tumours from a mouse xenograft model to study the expression of specific metabolic proteins in relation to the tumour responsiveness to radiation therapy. Our results isolated 9 metabolic proteins from differentially responsed to RT in CRC tumours which belong to some essential biochemical pathways such as glycolysis (PGK1, PGAM1, ENO1, PKM, TKT), ammonia detoxification (GLUD1), carcinogenesis (LDHA, GAPDH), and drug responses (MDH2). To warrant our findings, we further validated the expressions of PGK1, GLUD1, LDHA, and GAPDH in the tumours. Figure 7.2 summarized the results of Chapter 6. Our results suggested that these proteins may be used as biomarkers for RT response and it could enhance the treatment outcomes of personalized RT in CRC.

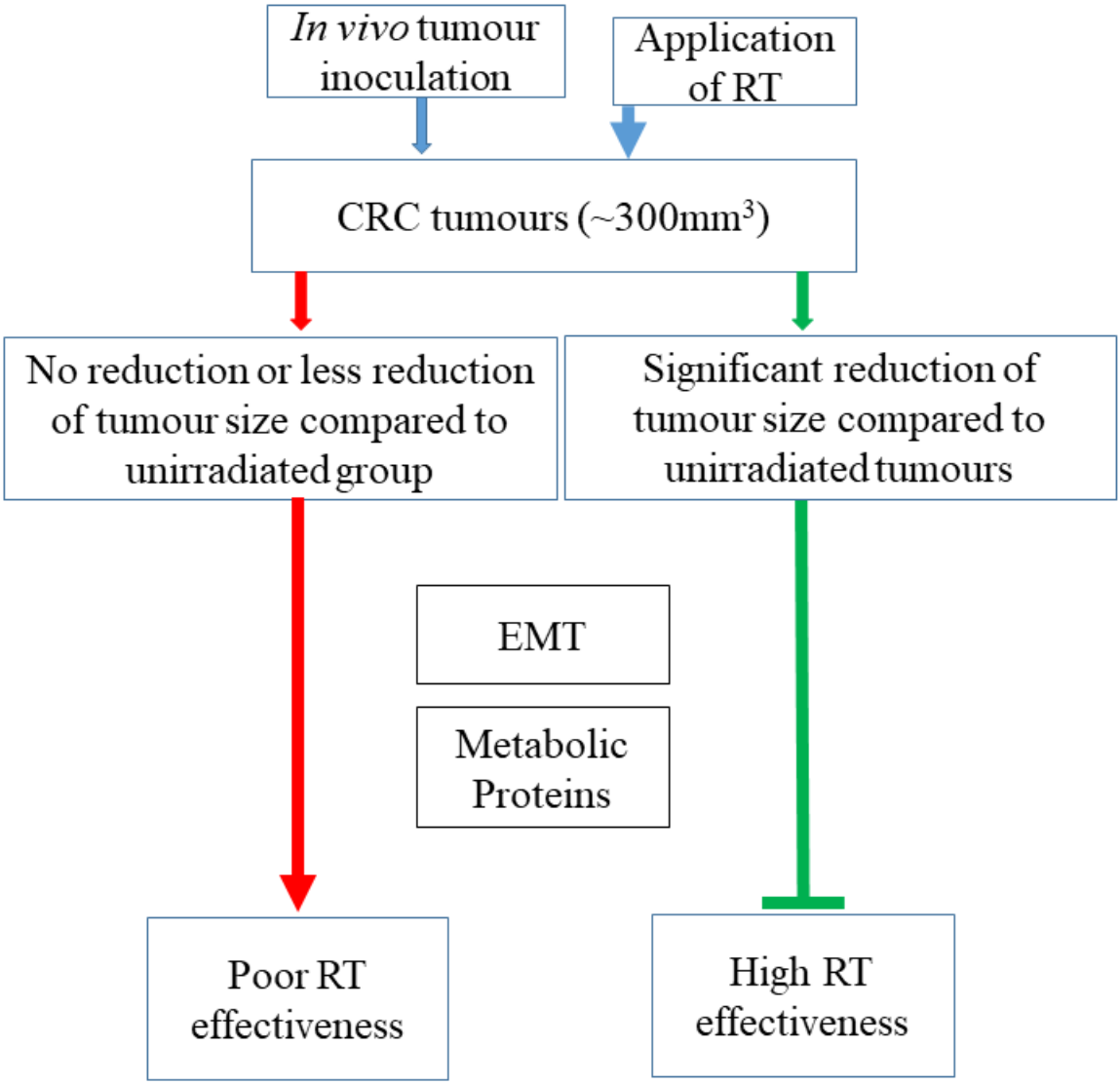


Figure 7.2. Metabolic proteins as RT-prognostic biomarkers.

Proteins are regulatory components of human body and cancer is the results of abnormalities in genetic alterations or environmental stress (Donnelly and Storchová, 2014). Importantly, cancer cells promote their metabolism and energy synthesis process to enable uncontrolled proliferation, growth, survival, and metastasis during stress condition (Phan et al., 2014, Yu et al., 2020a). RT effectively reduce tumour cells growth, proliferation, migration, and metastasis by DNA damage, affecting plasma membrane, and cellular signaling transduction such as EGFR, MAPK, PI3K, AKT, and HIF (Wang et al., 2018b). In addition, the advance in RT over the past decades along with molecular biology revealed that genes and proteins are key regulatory components in cell death process induced by RT (Wahba et al., 2016, Wang et al., 2018b). Therefore, restricting metabolism and metabolic pathways could be a potential targets in reducing the malignancies. Taken together, our study isolated 9 metabolic proteins that is deregulated with effective RT in CRC tumours. Therefore, it might be used as potential RT biomarkers in CRC and further investigation is needed to translate our findings into clinical settings.

Slightly different from the approach proposed for the use of lncRNAs as cancer biomarkers, we propose to collect tumour samples after the patients have received several doses of RT and process the samples for proteomics studies to predict the responsiveness to RT. This may provide information for the oncologists to adjust the CRC management and the radiation therapist to design new plans for further treatments.

7.3 Limitations and future works

Despite the well-established screening and treatment protocols, the mortality of CRC remains high and there is a recent trend to develop personalised treatment because not all patients can benefit from the same treatment regime. It has been demonstrated that predictive tests for tumour characteristics may facilitate the design of individualised RT to further improve the treatment

outcome and reduce treatment related complications. However, there is no biomarkers for CRC prognosis nor responsiveness to radiation. In the current study, we have proposed some biomarkers for CRC however it has some limitations. First of all, our lncRNAs experiments were performed on cell lines *in vitro.* The translation of CASC9 into patient samples was verified by gene expressions in open access platform but the translation of RAMS11 cannot be verified because this lncRNA is too new. Secondly, we have silenced CASC9 and RAMS11 in HCT-116 and SW480 to probe for the molecular mechanisms but we have not performed any animal studies on these two lncRNA. As for clinical application, we are very far from developing CASC9 and RAMS11 for diagnosis or treatment. We have detected their expression in the tumours but there is not information on their expression in the circulation. Further verification of the expression of CASC9 and RAMS11 in the blood of CRC patients may facilitate their applications as CRC biomarkers.

Moreover, we have performed cell experiments and WBt studies to investigate several carcinogenesis pathways including autophagy, apoptosis, mTOR and EMT. There are alternative methods for studying the same pathways for in depth analysis. For example, we may perform mCherry-GFP-LC3 assay for autophagic flux and Annexin V/Propidium Iodide flow cytometry or Tunnel assay for apoptosis. In addition, our study may extend to the study of more EMT proteins such as FSP1, HSP47, and ZO1.

To verify our findings, we may study carcinogenesis in tissue specific knock out mice. Then, to study how CASC9 and RAMS11 lead to autophagy, we may perform RNA pull-down assay or RNA immunoprecipitation assays.

Similar to other biomarkers study, a standard protocols need to be established and followed for sample preparation, storage, and RNA isolation from the biological fluids or tumours. Furthermore,

appropriate quantification techniques, quality of findings, sensitivity of the techniques, and precise analysis of CRC-associated lncRNAs may warrant the findings. Additionally, using universal control in all instrumental set up may lead to improve the sensitivity, reliability, and accuracy of the measurements. Besides, the large sample cohorts are another predominant factors in developing lncRNAs based biomarkers in CRC. For the current study, the most important limitation is the absence of *in vivo* study. Theoretically, it may be possible to induce or suppress lncRNAs in CRC cell lines to reduce carcinogenesis and metastasis however, extensive research still need to work for the next leap of technology to reach this goal.

Regarding the study on RT responsiveness, our study is limited to the xenograft generated by using a single cell lines HT-29. The *in vivo* experiments may be repeated using different CRC cell lines with more mice. Besides, it is important to verify the expression of our proposed markers in patients sample and determine their association with tumour size and treatment outcome after RT.

In this project, we first focus on the effect of radiation in CRC tumours and we will perform more experiments applying chemoradiation in our future projects. One concern is that the animal become quite weak after irradiation of 15Gy. Therefore, the chemotherapy dose titration needed to be handled carefully.

To overcome the lack of patient samples in this project, we have used online databases to check the expression of our shortlisted lncRNA and metabolic proteins. We used the default settings to plot all the graphs and survival curve. In fact, it is possible to extend the study by downloading the databases and perform big data analysis to verify the association of our shortlisted candidates with other parameters such as cancer stages and clinical outcomes. Unfortunately, there was no information on radio-responsiveness in all the databases available.

7.4 Conclusion

This thesis primarily demonstrated the roles of oncogenic lncRNAs CASC9 andRAMS11 in CRC progression and metastasis. This study revealed that CASC9 and RAMS11 expressions are significantly upregulated in CRC cells compared to adjacent normal cells where upregulation associated with progression of CRC. Silencing of CASC9 and RAMS11 reduce carcinogenesis through the induction of autophagy, activating AMPK, dephosphorylating AKT/mTOR and downregulating EMT process. The silencing of RAMS11 significantly induced cellular apoptosis process in tumour cells. We propose that CASC9 and RAMS11 expression may be served as novel potential biomarkers in CRC.

We have also isolated 9 metabolic proteins belongs to some essential biochemical pathways such as glycolysis, citric acid cycle, tricarboxylic acid cycle where some are associated with carcinogenesis, and drug responses. These proteins are significantly downregulated along good responding post RT CRC tumours. To warrant our findings, we further validated the expressions of those proteins from public databases and performed WBt for selected proteins. The results indicated that 9 proteins may combinedly be used as potential RT biomarkers in CRC, and further research may translate their use in clinical settings to improve RT outcomes for identical patients.

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