ELUCIDATION OF CIRCULAR RNA (CIRC007667 AND CIRC037236) ROLES IN COLORECTAL CANCER PATHOGENESIS

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ELUCIDATION OF CIRCULAR RNA (CIRC007667 AND CIRC037236) ROLES IN COLORECTAL CANCER PATHOGENESIS

by

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LIST OF SYMBOLS AND ABBREVIATIONS

ATCC	American Type Culture Collection
CDK2	cyclin-dependent kinase 2
ceRNAs	competitive endogenous RNA
circRNAs	circular RNAs
ciRNAs	intronic circRNAs
CME	complete mesocolic excision
CRC	colorectal cancer
CSC	cancer stem cells
CVD	cardiovascular diseases
ecircRNAs	Exonic circRNAs
elciRNA	Both exonic and intronic sequences
EMT	epithelial-to-mesenchymal transition
HIPEC	hyperthermic intraperitoneal chemotherapy
IHC staining	immunohistochemical staining
MBL	Muscleblind
mRNAs	messenger RNAs
PHLDA3	Pleckstrin homology-like domain family A, member 3
PKM2	M2 isoform of pyruvate kinase
PSMC3	proteasome 26S subunit, ATPase 3
p21	cyclin-dependent kinase inhibitor 1
QKI	Quaking

RBPs	compete RNA binding proteins
RCA	rolling circle amplification
RI	RNA immunoprecipitation
snRNPs	small nuclear ribonucleoproteins
TNM	tumour-node-metastasis
µL	Microlitre
µM	Micromolar
XXMU	XinXiang Medical university
17β-HSD1	17β-Hydroxysteroid dehydrogenase 1
<	Less than
>	Greater than
%	Percentage
× g	Relative centrifugal force
°	Degree
°C	Degree Celsiusu

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PENCIRIAN PERANAN RNA LINGKARAN (CIRC007667 DAN CIRC037236) DALAM PATOGENESIS KANSER KOLOREKTAL

ABSTRAK

Kanser kolorektal (CRC) merupakan kanser ketiga yang paling kerap didiagnosis di seluruh dunia dan penyebab kematian berkaitan kanser yang keempat tertinggi. RNA lingkaran (circRNA) adalah molekul RNA unik dengan struktur gelung tertutup, tanpa polariti 5′ ke 3′ dan ekor poli-adenilasi. Walaupun berpotensi, peranan circRNA dalam CRC masih kurang difahami. Kajian ini menyelidik peranan circ007667 dan circ037236 dalam CRC dan meneroka potensi mereka sebagai sasaran molekul untuk intervensi. Kaedah penjujukan pemprosesan tinggi telah mengenalpasti circRNA yang diekspreskan secara berbeza dalam pesakit CRC. Perbezaan ekspresi ini telah disahkan menggunakan PCR masa nyata kuantitatif (qPCR) dalam kedua-dua sampel tisu dan titisan sel CRC. Circ007667 kawal-atur-menurun digabungkan ke dalam vektor pcDNA3.1-CMV-circRNA-EF1-ZSGreen untuk menghasilkan model titisan sel peroleh-fungsi. Sebaliknya, model nyahfungsi dihasilkan dengan menurunkan ekspresi circ037236 kawal-atur-menaik. Kemudian, dengan menggunakan kaedah qPCR, tahap ekspresi circ007667 dan circ037236 dianalisis dalam titisan sel HCT116 dan SW1463 yang telah ditransfeksi. Percambahan sel dinilai menggunakan ujian MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)), manakala sitometri aliran digunakan untuk menentukan kadar

apoptosis dan kitaran sel. Migrasi sel diukur dengan ujian calar luka, dan ujian pelapor dual-luciferase mengesahkan mekanisme circRNA dalam CRC. Keputusan menunjukkan peningkatan ekspresi yang signifikan bagi circ037236 ($p<0.01$) dan penurunan circ007667 yang ketara ($p<0.05$) dalam tisu CRC dan titisan sel. Model peroleh-fungsi dan nyahfungsi menunjukkan bahawa circ007667 menyekat percambahan sel, peralihan kitaran sel, pertumbuhan, dan metastasis, manakala circ037236 menggalakkan proses-proses ini. Secara mekanistik, circ007667 meningkatkan penindas tumor PHLDA3 dengan menghalang miR-6510-5p, manakala circ037236 bertindak sebagai span untuk miR-6842-3p dan mempengaruhi aktiviti HSD17B1. Sebagai kesimpulan, kajian ini adalah yang pertama, yang menyelidik peranan circ007667 dan circ037236 dalam CRC secara komprehensif, sekaligus menekankan potensi mereka sebagai sasaran terapeutik untuk pengesanan dan rawatan CRC.

ELUCIDATION OF CIRCULAR RNA (CIRC007667 AND CIRC037236) ROLES IN COLORECTAL CANCER PATHOGENESIS

ABSTRACT

Colorectal cancer (CRC) ranks as the third most frequently diagnosed cancer worldwide and the fourth leading cause of cancer-related deaths. Circular RNAs (circRNAs) are unique RNA molecules with a closed-loop structure, lacking 5′ to 3′ polarity and a poly-adenylated tail. Despite their potential, the role of circRNAs in CRC remains poorly understood. This study investigates the roles of circ007667 and circ037236 in CRC, exploring their potential as molecular targets for intervention. High-throughput sequencing identified differentially expressed circRNAs in CRC patients, which were validated using quantitative real-time PCR (qPCR) in both CRC tissues and cell lines. The down-regulated circ007667 was incorporated into a pcDNA3.1-CMV-circRNA-EF1-ZSGreen vector to create a gain-of-function cell line model. Conversely, a loss-of-function model was established by knocking down the up-regulated circ037236. Expression levels of circ007667 and circ037236 were analysed in transfected HCT116 and SW1463 cell lines using qPCR. Cell proliferation was assessed using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)) assay, while flow cytometry was employed to evaluate apoptosis rates and cell cycle. Cell migration was measured with a wound scratch test,

and a dual-luciferase reporter assay validated the mechanisms of circRNAs in CRC. Results showed significant upregulation of circ037236 ($p<0.05$) and downregulation of circ007667 ($p<0.01$) in CRC tissues and cell lines. Gain-of-function and loss-of-function models demonstrated that circ007667 suppresses cell proliferation, cell cycle transition, growth, and metastasis, whereas circ037236 promotes these processes. Mechanistically, circ007667 enhances the tumour suppressor PHLDA3 by inhibiting miR-6510-5p, while circ037236 acts as a sponge for miR-6842-3p, affecting HSD17B1 activity. In conclusion, this study is the first to comprehensively investigate the roles of circ007667 and circ037236 in CRC, highlighting their potential as therapeutic targets for CRC detection and treatment.

CHAPTER 1
INTRODUCTION

1.1 Background

The research explores a relatively novel field that has emerged, focusing on the utilisation of circular RNA (circRNAs) for the diagnosis and treatment of colorectal cancer. circRNAs have recently been identified as a novel type of covalently closed transcripts generated by alternative, noncanonical pre-mRNA back-splicing events. Globally, circRNAs research in colorectal cancer (CRC) has gained significant attention in recent years. Studies have shown that circRNAs play crucial roles in various biological processes, including tumourigenesis, metastasis, immunity and drug resistance (Chen *et al.*, 2023; Ding *et al.*, 2023; Fang *et al.*, 2023; Li *et al.*, 2023). They exhibit unique characteristics such as stability, conservation, and tissue-specific expression patterns, making them attractive candidates for diagnostic and therapeutic applications. Chinese researchers have contributed significantly to the field, conducting studies to identify differentially expressed circRNAs in colorectal cancer tissues, exploring their potential as biomarkers for early detection and prognosis. Moreover, investigations have been conducted to elucidate the functional roles of circRNAs in colorectal cancer progression, including their involvement in regulating cancer-related pathways and molecular mechanisms (Song *et al.*, 2023; Xiong *et al.*, 2023).

In Malaysia, circRNAs research in colorectal cancer is gradually emerging. Research efforts have primarily centred on understanding the expression profiles and the regulatory networks driven by circRNAs in cancer stem cells (CSC) -enriched CRC spheroid cells, exploring their potential clinical implications, and investigating their roles in tumour development and progression (Rengganaten *et al.*, 2020). In another research, a chemoresistant cell line model was established, and circRNAs profiling was performed using microarray technology, to identify the distinctively expressed circRNAs between chemoresistant and chemosensitive CRC cells (Abu *et al.*, 2019).

Overall, both domestic and foreign research backgrounds demonstrate a growing recognition of circRNAs as promising targets in colorectal cancer research. While recent studies have contributed to our accelerated understanding of the roles and potential applications of circRNAs in diagnosing, treating, and managing colorectal cancer, there remain several uncharted areas in circRNAs research within this context. To address these gaps, it is imperative to encourage more scholars to develop into further exploration and research in this field.

1.2 Problem statement

Existing therapies for colorectal cancer (CRC) face significant limitations, such as the development of resistance to treatments, limited efficacy in certain genetic subtypes, significant toxicity and side effects, and challenges in treating metastatic disease. Additionally, tumour heterogeneity complicates treatment, cancer stem cells

often evade current therapies leading to relapse, and the lack of predictive biomarkers hampers personalized treatment approaches. Furthermore, the tumour microenvironment can influence treatment response. Addressing these shortcomings requires ongoing research to identify new molecular targets, develop more effective and less toxic therapies, and implement personalized medicine approaches.

The naming and annotation of circRNAs lack standardisation, making it challenging to compare findings across different studies. This can hinder the consolidation and interpretation of circRNAs-related data and limit the accuracy and reproducibility of research findings.

The precise mechanisms underlying circRNAs biogenesis and regulation are not yet fully understood. Investigating the factors and pathways involved in circRNAs formation, regulation, and degradation will provide valuable insights into their biological functions and potential therapeutic applications.

Despite the growing interest in circRNAs, our knowledge of their exact functions and mechanisms in colorectal cancer remains limited. Further research is necessary to comprehensively understand the roles of specific circRNAs and their impact on tumourigenesis, progression, and treatment response. While multiple studies have identified differentially expressed circRNAs in colorectal cancer, the functional significance and clinical relevance of many circRNAs remain largely unexplored. Additional functional studies and extensive clinical validation are required to establish the diagnostic, prognostic, and therapeutic potential of circRNAs in colorectal cancer.

In short, addressing these limitations will be crucial for advancing circRNAs research in colorectal cancer and harnessing the full potential of circRNAs as diagnostic tools, therapeutic targets, and prognostic indicators.

1.3 Study aims and objectives

In line with the National Strategic Plan for Cancer Control Programme (NSPCCP) 2021-2025, which covers all aspects of cancer control including early detection, diagnosis, prevention and treatment, rehabilitation, palliative care as well as traditional and complementary medicine (T&CM) and research, this study was carried out to elucidate the functions and roles of differentially expressed circRNAs in the development of CRC. This in turn leads to future potential and promising avenue in developing circRNAs as a biomarker for early detection and diagnosis of CRC as well as potential molecular intervention in the treatment of the disease.

The specific objectives of this study are:

1. To identify the expression profile of circRNAs in CRC tissue samples;
2. To validate the differential expression of target circRNAs in CRC tissues and CRC cell lines;
3. To establish the selected circRNAs lost-of- and gain-of-function cell line model systems (HCT116 and SW1463 cell lines);
4. To investigate the functions and mechanisms of selected circRNAs in CRC model cell lines.

1.4 Thesis contents

The title of this thesis is “Elucidation of Circular RNA (circ007667 and circ037236) Roles in Colorectal Cancer Pathogenesis”. This thesis comprises five chapters, starting with the introduction in the first chapter, followed by the literature review in the second chapter. The third chapter details the experimental methods and materials used in this study, while the fourth chapter presents the results. Lastly, the final chapter encompasses the discussion and conclusion.

The complete PHD research was carried out in both AMDI, USM, Penang, Malaysia and XXMU, XinXiang, China. Extensive literature review was conducted in the initial stage, leading to the determination of the research direction, content, and plan. The whole experiment involved the collection of tissue samples from CRC patients, followed by sequencing, data analysis, and extraction of results from a substantial volume of sequencing data. Additionally, the sequencing results were validated through qPCR conducted on both tissue samples from CRC patients and CRC cell lines. The research primarily centred around analysing the expression variations of two specific targets: an up-regulated circ037236 and a down-regulated circ007667. The roles of circRNAs were examined by conducting various assays including cell proliferation, cell cycle, apoptosis, and migration assays. Moreover, the study delved into the exploration of underlying mechanisms by employing tools such as TargetScan and MiRanda to deduce the microRNA and downstream mRNA

associated with the target circRNAs. The relationship between these elements was further validated through dual-luciferase reporter assay.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Colorectal cancer (CRC) is globally recognised as the third most prevalent form of cancer and the fourth major contributor to cancer-related fatalities (Ferlay et al., 2015; Torre et al., 2015). According to projections, there is expected to be a 60% increase in the global prevalence of CRC by the year 2030. This surge would lead to over 2.2 million new cases and 1.1 million fatalities (Arnold et al., 2017). Colorectal cancer is the second most common cancer in Malaysia and contributed to 13.5% of all new cancer cases diagnosed in 2012-2016 (NSPCCP). The survival outcomes of CRC heavily rely on the disease's stage upon diagnosis. Generally, 5-year survival rates for stage I and II are 90% and 80% respectively, while stage III ranges from 30-60%, and stage IV is typically around 5-10% (Haggar et al., 2009). Presently, a significant proportion of individuals who have been diagnosed with CRC are at stage III and IV, which not only correlates with unfavorable prognosis but also diminished the quality of life for these affected patients (Wan Puteh et al., 2013). Circular RNAs (circRNAs) possess a distinct structure known as a closed-loop, which sets them apart from other RNA molecules. They differ from typical RNAs in that they lack the conventional 5′ to 3′ polarity and do not have a poly-adenylated tail. Despite numerous investigations on circRNA in CRC, the underlying mechanism remains relatively unclear.

2.2 Colorectal cancer (CRC)

Colorectal cancer (CRC), referred to as bowel cancer, colon cancer, or rectal cancer, is characterized by the uncontrolled growth of cells in the colon or rectum. The colon, also known as the large intestine or large bowel, is responsible for waste processing, while the rectum serves as the connecting pathway between the colon and the anus. The latest research progress on CRC will be summarized, including risk factors, molecular biology, diagnosis, treatment, and prevention of CRC. In this review, we will summarise the latest research progress on CRC, including risk factors, molecular biology, diagnosis, treatment, and prevention of CRC.

2.2.1 Risk factors of CRC

Various factors bring about an individual's risk of developing CRC. These risk factors encompass age, race and ethnicity, family history, diet, lifestyle, certain medical conditions, and genetic mutations. Age is the most important risk factor, with most cases occurring in individuals over 50 years of age (Amersi *et al.*, 2005). Current evidence supports the notion that males exhibit a greater susceptibility to colorectal cancer (CRC) in comparison to females (Murphy *et al.*, 2011). CRC incidence rates vary among different racial and ethnic groups. African Americans have the highest CRC rates in the United States, followed by Caucasians, Hispanics, Asian Americans, and Native Americans (Jackson *et al.*, 2016; Carethers, 2021). A personal or family history of CRC or colorectal polyps, particularly in first-degree relatives, improves the risk of developing CRC (Amersi *et al.*, 2005). Lifestyle factors (Aleksandrova *et al.*, 2014), including diet high in red and processed meats, low in fiber, sedentary behavior,

smoking (Botteri *et al.*, 2020), and excessive alcohol consumption (Fedirko *et al.*, 2011), have also been linked to a higher likelihood of developing CRC. Moreover, certain medical conditions, such as inflammatory bowel disease (Munkholm, 2003; Shah *et al.*, 2022), colorectal polyps (Delavari *et al.*, 2014), have been associated with an increased risk of CRC.

2.2.2 Molecular biology of CRC

The development and progression of CRC are significantly influenced by molecular biology. Numerous genetic and epigenetic changes have been discovered in CRC, encompassing mutations in tumour suppressor genes, oncogenes, and DNA mismatch repair genes (Gryfe *et al.*, 1997). The presence of this mutation could potentially enable a developing tumour with several genetic changes to avoid cell cycle arrest and apoptosis. Furthermore, DNA sequences may undergo hypomethylation or hypermethylation, leading to changes in gene expression without any nucleic acid mutations (Fearon, 2011; Bogaert *et al.*, 2014). According to a study, 75% of the total cases exhibited the expected high microsatellite instability, often accompanied by hypermethylation and silencing of the MLH1 gene. The remaining 25% had somatic mutations in mismatch repair genes and polymerase ε (POLE) (Muzny *et al.*, 2012). These alterations lead to the dysregulation of key cellular pathways, including the Wnt/β-catenin and MAPK pathways, resulting in uncontrolled cell growth and proliferation.

2.2.3 Diagnosis of CRC

Diagnosis of CRC is typically based on a combination of physical examination, imaging, and laboratory tests. Colonoscopy and biopsy are widely regarded as the gold standard for screening and diagnosing CRC since they enable the identification and removal of precancerous lesions as well as early-stage tumours (Świderska *et al.*, 2014). Moreover, several non-invasive tests, such as stool-based tests, blood tests, and imaging tests, have been developed for CRC screening and diagnosis. For instance, different indicators for CRC, including CEA, CA-19-9, TPS, TAG-72, and lysosomal hydrolases, have been identified and are currently utilized as routine clinical practices (Świderska *et al.*, 2014).

2.2.4 Treatment of CRC

The treatment of CRC is determined by the stage and location of the tumour and may encompass surgical procedures, chemotherapy, radiation therapy, targeted therapy, and immunotherapy (Nordlinger *et al.*, 2009; Xie *et al.*, 2020). Surgery is the primary treatment for early-stage CRC, some of these strategies consist of complete mesocolic excision (CME), treatments for metastatic disease, hyperthermic intraperitoneal chemotherapy (HIPEC), and surgical techniques designed to address colorectal cancer recurrence. While chemotherapy and targeted therapy are commonly used to treat advanced-stage CRC, recent successes with anti-EGFR agent cetuximab and anti-angiogenesis agent bevacizumab have led to the emergence of new agents that block critical pathways and immune checkpoints at an unprecedented rate (Piawah *et al.*, 2019; Xie *et al.*, 2020). Immunotherapy, particularly immune checkpoint

inhibitors, has shown promising results in treating CRC, especially in microsatellite instability-high tumours. Drugs targeting CTLA-4 (ipilimumab, tremelimumab) and PD-1/PD-L1 (nivolumab, pembrolizumab, cemiplimab, atezolizumab, durvalumab, avelumab) have been tested in various clinical trials (Rzhevskiy *et al.*, 2021). There is a growing body of encouraging findings in preoperative and postoperative treatments, such as chemotherapy, chemoradiotherapy, and targeted therapy. But identifying novel biomarkers appears to be critical for making further advancements in the treatment outcomes of patients with CRC (Matsuda *et al.*, 2018).

2.2.5 Prevention of CRC

Prevention of CRC is primarily based on lifestyle modifications and screening. An example of this is that consuming a diet rich in fiber, fruits, and vegetables while minimizing the intake of red and processed meat has been linked to a decreased likelihood of developing CRC (Amersi *et al.*, 2005). Moreover, regular physical activity and avoidance of tobacco and excessive alcohol consumption have been linked to a lower risk of CRC (Fedirko *et al.*, 2011; Aleksandrova *et al.*, 2014; Botteri *et al.*, 2020). In addition, CRC screening, starting at age 45 for individuals at average risk, can help detect precancerous lesions and early-stage tumours and reduce the incidence and mortality of CRC (Amersi *et al.*, 2005).

In conclusion, CRC is a major public health problem that can be prevented and treated through a combination of lifestyle modifications and screening. In recent research advancements, multiple risk factors for CRC have been identified, new diagnostic and screening tools have been developed, and significant progress has been

made in the treatment and prevention of CRC. Continued research is essential to enhance our comprehension of the underlying mechanisms of CRC and to devise novel strategies for the prevention, diagnosis, and treatment of this disease.

2.3 Circular RNAs (circRNAs)

Although circRNAs transcripts were initially discovered in the early 1990s (Nigro *et al.*, 1991), our understanding of these molecules has remained limited until recent years. Circular RNA, also known as circRNAs, is a single-stranded RNA that differs from linear RNA in that it creates a continuous loop through covalent closure. This type of RNA joins the 3' and 5' ends that are typically found in RNA molecules, and it is a covalently closed loop structure without 5′to 3′polarity and poly-adenylated tail (Salzman *et al.*, 2012). Due to its closed structure, circRNAs is more resistant to nucleases, which are enzymes that often target the ends of linear RNA molecules. circRNAs exhibits greater stability compared to linear RNA, resulting in reduced susceptibility to degradation (Jeck *et al.*, 2013). CircRNAs is known to be present in significant numbers and exhibits stability within the cytoplasm of eukaryotes (Salzman *et al.*, 2012). Moreover, the abundance of circRNAs can sometimes exceed 10 times that of the corresponding linear mRNA. The researchers investigating human fibroblasts uncovered more than 25,000 distinct RNA variants that contain noncollinear exons, resulting in a "backsplice" conFiguration. These variants were consistently found to be enriched through the exonuclease-mediated degradation of linear RNA (Jeck *et al.*, 2013).

2.3.1 The biogenesis of circRNAs

Extensive evidence has demonstrated the significant involvement of circRNAs in diverse biological processes, including RNA splicing, gene regulation, and protein synthesis. However, the biogenesis of circRNAs is still not fully understood. This review aims to provide a comprehensive summary of the current understanding regarding the biogenesis of circRNAs.

2.3.1(a) Alternative back-splicing

Emerging research findings have indicated that alternative back-splicing might play a regulatory role in the biogenesis of circRNAs (Zhang *et al.*, 2016; Feng *et al.*, 2019). For example, the inclusion or exclusion of certain exons in pre-mRNA can affect the formation of circRNAs derived from those exons. This suggests that the biogenesis of circRNAs are tightly linked to the splicing process. The formation of circRNAs occurs through a back-splicing event, involving the joining of a downstream 5' splice site with an upstream 3' splice site. This process leads to the circularization of the RNA molecule, facilitated by RNA-binding proteins, such as Quaking (QKI) and Muscleblind (MBL). Quaking (QKI) is a member of the STAR (Signal Transduction and Activation of RNA) family of RNA-binding proteins, which play crucial roles in various aspects of RNA metabolism, including splicing, stability, and translation. Similarly, Muscleblind (MBL) is an essential RNA-binding protein involved in RNA processing events, including alternative splicing and the regulation of RNA stability(Ashwal-Fluss *et al.*, 2014; Conn *et al.*, 2015). The donor splice site, also known as the 5' splice site, and the acceptor splice site, also known as the 3' splice

site, refer to specific sequences located at the 5' and 3' ends of an intron, respectively. These sequences are recognized by the spliceosome, a complex involved in RNA splicing. After recognition by the spliceosome, the branch point, a downstream sequence, causes a nucleophilic attack on the 5' splice site sequence, resulting in the formation of a circular structure known as a lariat. Following the formation of a lariat structure, the free 5' exon initiates an attack on the 3' splice site, resulting in the joining of the two exons and the subsequent release of the intron lariat structure. Subsequently, the intron lariat undergoes debranching and is rapidly degraded (Reece, 2011).

The location of the back-splicing event determines the formation of various types of circRNAs. Exonic circRNAs (ecircRNAs) are formed from exonic regions of pre-mRNA (Memczak *et al.*, 2013), while intronic circRNAs (ciRNAs) are derived from intronic regions (Zhang *et al.*, 2013). There are also circular RNA transcripts (elciRNA) that contain both exonic and intronic sequences, and intergenic circRNAs that are derived from regions between genes (Figure2.1) (Salzman *et al.*, 2013). Based on available literature, the predominant mechanism of action for ecircRNAs is believed to be the microRNA (miRNA) "sponge" mechanism. This concept was initially proposed by Memczak et al., who identified 63 binding sites for microRNA-7 on CDR1as, thereby classifying CDR1as as a " miRNA sponge". EcircRNA is capable of boosting the expression of miRNA target genes by capturing miRNA molecules through adsorption. In contrast to ecircRNAs, circRNAs that contain introns (known as ciRNAs or EIciRNAs) are typically located in the nucleus and have a regulatory role in gene transcription (Zhang *et al.*, 2013; Li *et al.*, 2015, 2017). The

discoveries made in this context revealed that EIciRNAs play a role in enhancing the expression of their parental genes through a cis-regulatory mechanism. This mechanism involves targeted RNA-RNA interactions between U1 small nuclear ribonucleoproteins (snRNPs) and EIciRNAs, providing a regulatory mechanism for transcriptional control. EIciRNAs primarily localize within the nucleus, where they engage in interactions with U1 snRNPs and facilitate the transcriptional activation of their parental genes (Li *et al.*, 2015, 2017).

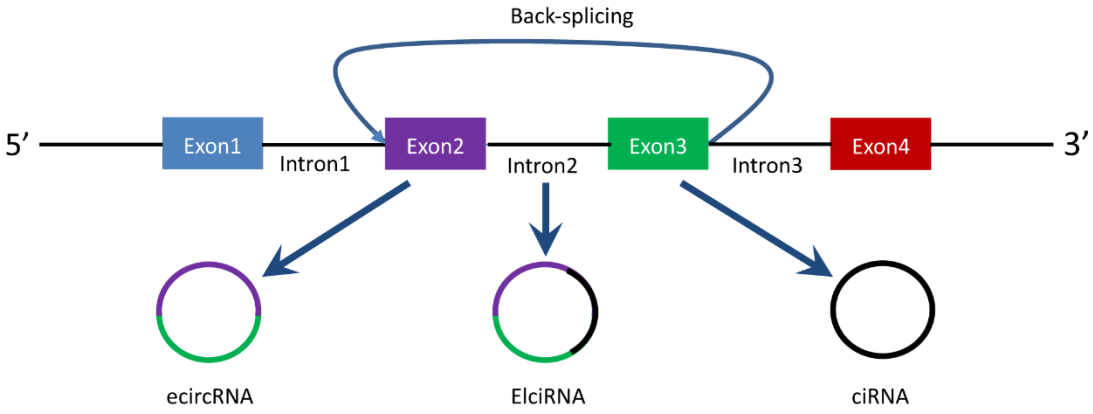

Figure2.1 Classification of circRNAs. ecircRNAs are formed from the exonic regions of pre-mRNA molecules. ElciRNAs, on the other hand, contain a combination of exonic and intronic sequences. Additionally, ciRNAs are circular RNAs that originate from intronic regions.

2.3.1(b) Regulation factors

The biogenesis of circRNAs is tightly regulated by various factors, such as RNA-binding proteins, splicing factors, and cis-acting elements present within the RNA molecule. One example of circRNAs biogenesis regulation is the involvement of the splicing factor FUS. FUS (Fused in Sarcoma) is a multifunctional RNA-binding protein that plays significant roles in various aspects of RNA metabolism, including splicing, transcription, and transport. FUS has been demonstrated to modulate

circRNAs production by binding to the introns surrounding the back-splicing junctions. This regulatory mechanism has been effectively replicated using synthetic constructs in experimental studies (Errichelli *et al.*, 2017). Furthermore, the presence of inverted repeat Alu elements within the RNA molecule has been observed to facilitate circularization through the formation of stable RNA structures (Jeck *et al.*, 2013; Zhang *et al.*, 2014). It is worth mentioning that the circularization of exons can be influenced by the competition between RNA pairing within individual introns or across adjacent introns. This mechanism is particularly fascinating since it can generate multiple circular RNA transcripts from a single gene. It occurs through the alternative formation of inverted repeated Alu pairs and the subsequent competition between them (Zhang *et al.*, 2014).

In conclusion, although the precise mechanisms underlying circRNA biogenesis are not yet fully elucidated, it is evident that this process is intricate and subject to regulation by a diverse range of factors. Additional research is necessary to gain a comprehensive understanding of the mechanisms governing circRNA biogenesis and to uncover the functional significance of circRNAs in diverse biological processes.

2.3.2 The functions of circRNA

CircRNAs have proven to be crucial players in gene regulation through their roles as miRNA sponges and as sequesters of RNA-binding proteins, among other functions. Moreover, they serve as valuable clinical markers and hold potential as molecular targets for various applications.

2.3.2(a) miRNA sponges

CircRNAs are also called competitive endogenous RNA (ceRNAs), one of the most well-known functions of circRNAs is their ability to act as natural miRNA sponges, which involves competitively binding to miRNAs and preventing them from binding to their target mRNAs. This results in a regulated expression of target genes and can have significant effects on various cellular processes, including cell proliferation, apoptosis, invasion, metastasis and differentiation (Hansen *et al.*, 2013; Memczak *et al.*, 2013; Lasda *et al.*, 2014). Numerous circRNAs, including circHIPK3 (Yan *et al.*, 2020), circCCDC66 (Mo *et al.*, 2022), and circPTK2 (Yang *et al.*, 2020; Jiang *et al.*, 2022), have been identified and characterized as miRNA sponges in relevant studies.

2.3.2(b) RNA-binding protein sequesters

Besides their function as miRNA sponges, circRNAs can play a pivotal role in gene regulation by sequestering various RNA-binding proteins. These proteins include RNA polymerase II, splicing factors, and transcription factors. By sequestering these proteins, circRNAs can exert regulatory control over gene expression at both the transcriptional and post-transcriptional levels. One illustrative example is the ectopic expression of circ-Foxo3, which forms a ternary complex by binding to cell cycle proteins such as cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (p21). This complex formation leads to the suppression of cell cycle progression, highlighting the regulatory role of circ-Foxo3 in controlling cellular processes. Under normal conditions, the interaction between CDK2 and cyclin A or

cyclin E is vital for the initiation of the cell cycle. However, the presence of p21 inhibits these interactions, effectively halting cell cycle progression. This regulatory mechanism ensures proper control and regulation of the cell cycle. Once the circ-Foxo3-p21-CDK2 ternary complex is formed, the function of CDK2 is arrested, resulting in the inhibition of cell cycle progression. This complex formation acts as a regulatory mechanism to halt the normal functioning of CDK2 and prevent the progression of the cell cycle (Du *et al.*, 2016). CircRNAs can additionally play a role in gene expression regulation by interacting with chromatin-modifying enzymes, such as EZH2 and PRC2, and affecting histone modification patterns (Mirzaei *et al.*, 2022). An example of circRNA-mediated regulatory effects can be observed in the case of ElciRNAs circEIF3J and circPAIP2, which exert cis regulatory effects on their respective parental genes EIF3J and PAIP2. To enhance gene expression, circEIF3J and circPAIP2 interact with key regulatory components such as RNA polymerase II (Pol II), U1 snRNP, and the promoters of their parental genes. These interactions contribute to the upregulation of gene expression by facilitating transcriptional processes and promoting the activity of the parental gene promoters (Li *et al.*, 2015, 2017).

2.3.2(c) Additional functions in biological processes

Furthermore, circRNAs have been implicated in a diverse of biological processes, including RNA splicing (Ashwal-Fluss *et al.*, 2014), protein synthesis (Chen *et al.*, 1995; Pamudurti *et al.*, 2017), and cellular differentiation (Di Timoteo *et al.*, 2020; Lin *et al.*, 2021). CircMbl, an MBL-derived circRNA, has a pivotal role in

regulating the expression of MBL protein isoforms in a tissue-specific manner. It exhibits unique functions in both cis and trans, leading to the regulation of gene expression and physiological processes in a tissue-specific manner. Depletion of MBL-C or circMbl results in contrasting behavioral phenotypes (Pamudurti *et al.*, 2022). CircRNAs have the ability to undergo cap-independent translation, which can occur through different mechanisms such as IRES-initiated pattern, MIRES-initiated pattern, and rolling circle amplification (RCA) (Wang *et al.*, 2022).

2.3.2(d) Clinical markers and molecular targets

In addition, circRNAs can compete RNA binding proteins (RBPs) for its mRNA targets, hence, alters gene expression (Barrett *et al.*, 2016). Recent studies have provided evidence suggesting that circRNAs hold diagnostic and prognostic potential as biomarkers for a range of diseases, including cancer, cardiovascular disease, and neurological disorders. CircRNAs have been identified as being dysregulated in cancer, suggesting their potential utility as diagnostic and prognostic biomarkers. The aberrant expression patterns of circRNAs in cancer hold promise for their application as valuable tools in the diagnosis and prognosis of cancer patients. Studies have found that circRNA is associated with colorectal cancer (Jin *et al.*, 2019), gastric cancer (Zhang *et al.*, 2019), bladder cancer (Zeng *et al.*, 2019), cardiovascular disease (Holdt *et al.*, 2018; Altesha *et al.*, 2019) and other diseases. In addition, circRNA is also associated with brain function (Piwecka *et al.*, 2017).

2.3.2(d)(i) CircRNAs and cardiovascular disease

Emerging research has demonstrated the involvement of circRNAs in cardiovascular diseases (CVD) and their potential as biomarkers and therapeutic targets. These findings highlight the significance of circRNAs in the pathogenesis of CVD and suggest their potential application in the development of diagnostic tools and targeted therapies for cardiovascular disorders.

CircRNAs have been found to be involved in cardiac development and maintaining normal cardiac function. They can regulate gene expression and signaling pathways critical for heart development and homeostasis. Dysregulation of specific circRNAs has been linked to cardiac abnormalities and heart disease (Holdt *et al.*, 2018; Li *et al.*, 2018; Zhang *et al.*, 2020). CircRNAs have been implicated in vascular biology and endothelial cell function. They can regulate processes such as angiogenesis, vascular smooth muscle cell proliferation, and endothelial cell activation. Dysfunction of circRNAs in these pathways can contribute to vascular disorders, atherosclerosis, and hypertension (Aufiero *et al.*, 2019; Jaé *et al.*, 2019).

CircRNAs have been implicated in modulating the inflammatory response and immune function in CVD (Chen *et al.*, 2019). They can regulate the expression of genes involved in immune cell activation, cytokine production, and inflammatory signaling pathways. Dysregulated circRNAs can contribute to chronic inflammation and immune system dysfunction associated with CVD (Ghafouri-Fard *et al.*, 2021; Yu *et al.*, 2021).

2.3.2(d)(ii) CircRNAs and neurological disorders

CircRNAs are involved in various stages of neuronal development, including neuronal differentiation, migration, and maturation. They participate in the regulation of key genes and signaling pathways critical for proper brain development (Van Rossum *et al.*, 2016). CircRNAs have been found to modulate neuronal function and synaptic plasticity, which are fundamental processes underlying learning, memory, and cognitive functions (You *et al.*, 2015). Dysregulation of specific circRNAs can disrupt these processes and contribute to neurological disorders such as Alzheimer's disease and autism spectrum disorders (Lee *et al.*, 2019; Zhang *et al.*, 2021).

CircRNAs show promise as diagnostic biomarkers and therapeutic targets for neurological disorders. Their stable presence in biofluids, such as cerebrospinal fluid and blood, makes them attractive candidates for non-invasive diagnostic tests. Additionally, targeting specific circRNAs through gene therapy or RNA-based therapies holds potential for developing novel treatments for neurological disorders.

2.3.2(d)(iii) CircRNAs and cancer

CircRNAs can function as regulators of gene expression in cancer cells. They can interact with miRNAs and sequester them, acting as miRNA sponges. By binding to miRNAs, circRNAs prevent their interaction with target messenger RNAs (mRNAs), leading to increased expression of target genes involved in cancer-related processes (Dai *et al.*, 2023; Zhai *et al.*, 2023). CircRNAs can also have both oncogenic and tumour-suppressive functions depending on the specific context (Li *et al.*, 2023). Some circRNAs promote cancer progression by enhancing proliferation, invasion, and

angiogenesis, while others exhibit tumour-suppressive effects by inhibiting these processes (Liu *et al.*, 2023; Dai *et al.*, 2023). Besides, circRNAs have been implicated in epigenetic regulation, influencing DNA methylation and histone modification patterns in cancer cells. They can participate in chromatin remodeling and affect gene expression through epigenetic modifications (Lin *et al.*, 2022; Wang *et al.*, 2022; H. Liu *et al.*, 2023).

In conclusion, circRNAs are emerging as important regulators of gene expression and play critical roles in a variety of biological processes. Further research is necessary to obtain a comprehensive understanding of the functional roles of circRNAs, as well as to explore their potential as therapeutic targets and diagnostic biomarkers for diverse diseases. By delving deeper into the mechanisms governing circRNA biology, we can uncover valuable insights that may contribute to the development of innovative therapeutic strategies and improved disease diagnostics. Although there has been progress in understanding circRNAs, the precise correlation between circRNAs and the progression of CRC has yet to be fully elucidated.

2.4 CircRNAs and CRC

CircRNAs have emerged as a fascinating area of research in the field of cancer biology. These RNA molecules, characterized by a covalently closed loop structure, have been found to play significant roles in various aspects of CRC cancer development and progression.

2.4.1 Abnormal expression of circRNAs in CRC

CircRNAs can exhibit specific expression patterns in CRC cancer. Some circRNAs are upregulated, while others are downregulated in cancer cells compared to normal cells. The observed changes in their expression levels indicate the potential of circRNAs as biomarkers for the diagnosis and prognosis of cancer. Advancements in gene sequencing technology have revealed that the expression of numerous circRNAs is dysregulated in colorectal cancer tissues, leading to the modulation of various genes, transcription factors, and signaling pathways. Notably, the TP53 gene, E2F1, and Wnt/β-catenin pathway, among others, have been implicated in the regulatory mechanisms of these up-regulated or down-regulated circRNAs. This expanding knowledge highlights the intricate involvement of circRNAs in colorectal cancer pathogenesis and provides valuable insights into potential therapeutic targets and biomarkers for the disease. (Chaudhary *et al.*, 2020; Chen *et al.*, 2019; Lai *et al.*, 2020). In this review, 67 studies were included, in which 51 articles reported on the up-regulated circRNAs and another 16 articles on the down-regulated circRNAs in CRC.

2.4.1(a) The upregulated circRNAs in CRC

A growing body of research indicates that circRNAs, functioning as oncogenes, play a significant role in the development and metastasis of colon cancer. These circRNAs have been found to contribute to tumour pathogenesis by promoting cell proliferation, migration, invasion, and angiogenesis (Chen *et al.*, 2020; Ma *et al.*, 2020). We summarised these circRNAs in Table 1, as shown below. Among these 51

RNAs, facilitates CRC progression by modulating different miRNA-mRNA axis, so they have the different mechanism when they perform similar functions.

In a study conducted by Ritu Chaudharya et al., RNA-seq data from three colorectal cancer cell lines (HCT116, RKO, and SW48) were analyzed. The researchers examined the effects of a DNA damaging agent on gene expression. Surprisingly, they observed that while numerous p53-induced mRNAs were strongly induced upon DNA damage, only a small number of circRNAs were upregulated from p53-induced genes. Notably, one of the circRNAs identified was circ-MDM2, derived from the MDM2 locus, which is known to be regulated by p53. Further investigations revealed that knocking down circ-MDM2 using specific siRNAs did not impact the levels of MDM2 mRNA or MDM2 protein. However, it resulted in increased basal p53 levels and led to growth defects both *in vitro* and *in vivo*. This finding suggests that circ-MDM2 functions as a novel therapeutic target derived from the MDM2 locus, suppressed p53 levels and inhibited cell cycle progression (Chaudhary *et al.*, 2020). In another study, it was discovered that the overexpression of circMYH9 contributes to the proliferation of CRC cells. This effect is achieved through the modulation of serine/glycine metabolism and redox homeostasis in a p53-dependent manner. The findings suggest that targeting circMYH9 and its associated pathway could be a promising therapeutic approach for the treatment of CRC (Liu *et al.*, 2021).

In a study conducted by Chen et al., a cohort of 70 CRC patients who underwent primary surgical resection and five CRC cell lines were examined. The researchers observed a significant increase in the expression of circ-001971 in both