Molecular Therapy
Nucleic Acids
Review

Emerging Epigenetic Regulation of Circular RNAs in Human Cancer

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Circular RNAs (circRNAs) are novel members of the noncoding RNA family. Their characteristic covalent closed-loop structure endows circRNAs that are much more stable than the corresponding linear transcript. circRNAs are ubiquitous in eukaryotic cells, and their functions are diverse and include adsorbing microRNAs (miRNAs; acting as miRNA sponges), regulating transcription, interacting with RNA-binding proteins, and translating and deriving pseudogenes. Moreover, circRNAs are associated with the occurrence and progression of a variety of cancers, acting as new biomarkers for early diagnosis to evaluate curative effects and patient prognosis. Here, this paper briefly describes the characteristics and functions of circRNAs, and it further concludes the relationship between circRNAs and human cancer.

Based on the potential of encoded proteins, the RNA family can be divided into two categories, coding RNAs and noncoding RNAs; noncoding RNAs include long noncoding RNA, riRNA, tRNA, nsRNA, and microRNA (miRNA). In recent years, RNA research has made great progress in the identification of noncoding RNAs, which are involved in a variety of biological processes.[1,2]

Circular RNAs (circRNAs) have nearly 30 years of history. They are a special class of noncoding RNAs derived from the back-splicing or exon skipping of pre-mRNAs. Unlike linear RNAs, circRNAs do not have a 5-cap and 3-poly(A) tails, which are produced by back-splicing exons, and the downstream 3-splicing donor is connected in reverse bond to the upstream 5-split acceptor.[3] circRNAs are stable due to their special circular structure, and they are not easily degraded by exonucleases, thus having a longer half-life. circRNAs are considered inert by-products of abnormally spliced linear RNAs.

With the emergence of high-throughput sequencing, an increasing number of circRNAs have been found in eukaryotic cells. Increasing evidence shows that the expression profiles of circRNAs in carcinoid tissues are different from those in normal tissues.[4,5] In addition, circRNAs have been reported to participate in a variety of cellular cancer-related physiological processes, including cancer initiation, progression, and metastasis.[6] Therefore, an in-depth analysis of circRNAs should help further clarify the epigenetic level of cancer-related mechanisms.

Biogenesis and Classification of circRNAs

According to their differences in the genome and constituent sequences, circRNAs can be divided into three categories: exon-derived circRNAs, intron-derived circRNAs, and circRNAs composed of exons and introns.[7–9] Three models are used to illuminate the possible formation of circRNAs: lariat-driven circularization, intron pairing-driven circularization, and RNA-binding protein-driven circularization (Figure 1). During exon skipping (cassette-on), the spliced intron lariat still reserves the skipped exon(s). A stable RNA circle can be produced when further splicing occurs before the lariat is decomposed by debranching enzymes.

Biogenesis of circRNAs

Lariat-Driven Circularization. Lariat-driven circularization is also known as the exon-skipping mechanism. The pre-mRNA partially folds during transcription, causing the 5′ splicing site (donor site) of the upstream intron to approach and attack the 3′ splicing site (receptor site) of the downstream intron, whereby the circRNA is formed by back-splicing of the folded region, while the remaining exons form a linear mRNA.[10,11] This is the mechanism for the formation of most circRNAs. For example, Kelly et al.[8] found that human umbilical vein endothelial cells stimulated with tumor necrosis factor α or tumor growth factor β contained a large number of circRNAs formed by lariat-driven circularization.

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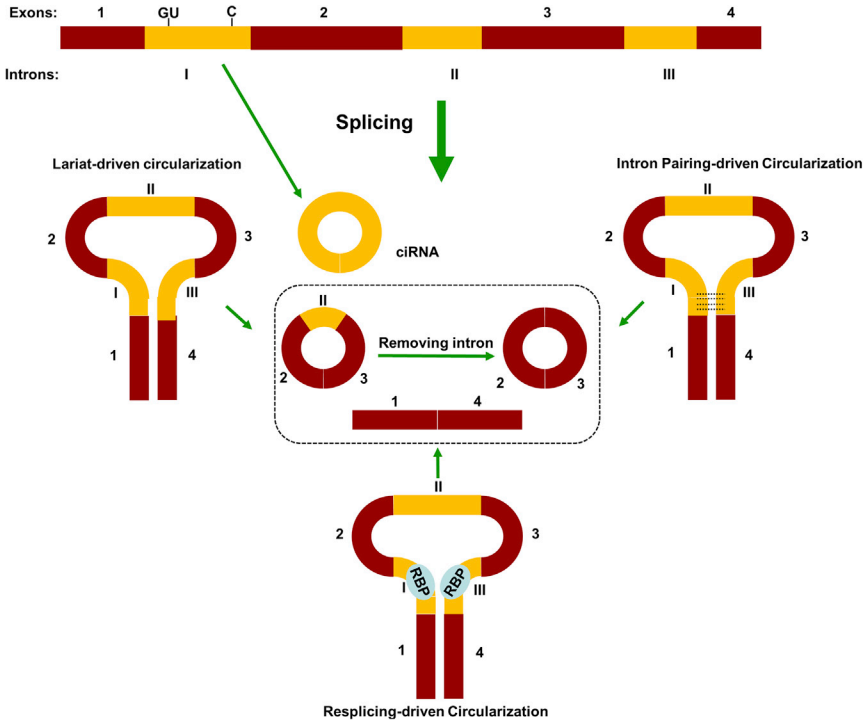

Figure 1. Biogenesis of circRNAs
Three models were considered to illuminate the possible formation of circRNAs. In lariat-driven circularization, a circRNA is formed by back-splicing of the folded region. In intron pairing-driven circularization, reverse complementary sequences on the flanks of introns mediate back-splicing to form a circRNA. In RNA-binding protein-driven circularization, RBPs shorten the distance between the donor site and the receptor site by binding to the introns on the flanks, promoting the circularization of exons.

Intron Pairing-Driven Circularization. Intron pairing-driven circularization is also known as the direct back-splicing mechanism. Reverse complementary sequences on the flanks of introns mediate back-splicing to form circRNAs. Flanking complementary sequences (especially Alu sequences) play a crucial part in exon circularization, and perfectly matched complementary sequences can promote the expression of circRNAs.[12,13] In this procedure, circRNAs can be divided into two patterns according to whether partial intron sequences are retained, namely, exonic circRNAs (EcircRNAs) from exons and circRNAs that coexist between intron and exon sequences (EIciRNAs).[14] Hsa-circ-POLR2A is a typical intron pairing-driven circRNA.[13]

RNA-Binding Protein-Driven Circularization. During RNA-binding protein-driven circularization, RNA-binding proteins (RBPs) can shorten the distance between the donor site and the receptor site by binding to the introns on the flanks, thus promoting the circularization of the exons. Muscleblind protein and quaking protein are two known RBPs that promote the formation of circMbl and circQKI, respectively.[15,16] Therefore, RBPs play a crucial role in the formation of some circRNAs.

Classification of circRNAs

EcircRNAs. There are two hypotheses for the formation of exon-derived circRNAs. The first hypothesis is that the pre-mRNA crosses an exon during transcription, the splicing enzyme then cleaves at both ends of the crossed exon, and the two ends are connected to form a closed-loop structure (lariat);[7] therefore, multiple circRNAs are generated by splicing. circRNAs can be derived from a single exon by back-splicing and can also be formed by exon splicing. Another hypothesis is that, during RNA transcription, the introns at both ends of the exons are base-paired, the downstream exon's 3′ end tail is connected to the upstream exon's 5′ end head, the downstream 3′ spliceosome is bound to the upstream 5′ splicing receptor, resulting in the binding of the two introns, and the cyclized exons are released as a circRNA.[8]

Many EcircRNAs contain exons that encode gene sequences and are normally spliced at standard splicing sites through the splicing copolymer mechanism. Genome-wide analysis of RNA sequencing (RNA-seq) data suggests that EcircRNAs are abundant in the mammalian transcriptome, and some EcircRNA sequences and their expression are conserved in evolutionary variation, revealing that they have cellular functions.[10,12,17] Specifically, EcircRNAs have been indicated to be much more steady than linear RNAs in plasma[13] and saliva,[14] suggesting that they may be diagnostic biomarkers.

Intron-Derived circRNAs. In contrast to EcircRNAs, intron-derived circRNAs (IciRNAs) have 3′∼5′ head-to-tail junction regions and differ in stability, subcellular localization, abundance, preservation, and function. IciRNAs are circularized on the chain of the branchpoints 2′∼5′, degenerating from the 3′ end to the branchpoint and avoiding detachment and degradation in a specific way; therefore, they are actually stabilized intron lariats.[15] Their synthesis requires an important site: a c-rich site containing 11 nt near the 5′ terminus, with a length of 7 nt, and a base-rich GU splicing site near the RNA-splicing branch site.[16]

EIciRNAs. Approximately 20% of EIciRNAs retain introns, and the retention of introns in the exons would make the circRNAs in this subclass more unique while retaining the functions of EcircRNAs and IciRNAs. Mainly located in the nucleus, EIciRNAs interact with U1 small nuclear ribonucleoprotein particle (snRNP) to promote the transcription of their parental genes. In the regulation of nuclear gene expression, EIciRNAs enhance the expression of parental genes in *cis* and emphasize the transcriptional regulation strategy through a specific RNA-RNA interaction between EIciRNAs and U1 small nuclear RNA (snRNA).[17,18]

Research Methods

Methods of Molecular Biology

The closed structure of circRNAs is highly stable and resistant to enzyme digestion; therefore, it can be preliminarily purified and identified by the following molecular biology methods.[19] (1) Most linear RNAs are degraded by exonuclease R, niacin phosphatase 5′-terminal exonuclease, and circRNAs are retained. Then, circRNA-specific primers are used for the quantitative analysis of the enzyme samples, which can be used to determine or quantify circRNAs before and after treatment.[19,20] (2) circRNAs have no polar structure at the end, and their migration rate in a cross-linked gel is slower than that of long linear RNAs. Compared with homologous gene transcription, nucleic acids have fewer circRNA sequences, and their migration rate in weakly cross-linked gels is slower. Therefore, circRNAs can be identified by northern blot analysis.[21,22] (3) Fluorescence *in situ* hybridization can be used to localize circRNAs at the subcellular level, and small interfering RNAs (siRNAs) or antisense oligonucleotides can be used to interfere with circRNA expression to verify the functions of circRNAs.[23,24]

High-Throughput Sequencing

Compared with traditional molecular biology methods, the combination of high-throughput sequencing and bioinformatics provides a shortcut for the discovery of new circRNAs with low abundance. circRNAs are generated by back-splicing, while the early RNA-seq algorithm is extremely inefficient in distinguishing back-splicing sites from the corresponding circle structures. Researchers have effectively improved the strategies and algorithms for sequencing analysis as follows: (1) assuming different forms of exon rearrangement, a circRNA candidate sequence boundary combination was constructed and then compared with the sequencing data;[25] (2) sequencing data are directly matched with the genome sequence through different sequence alignment algorithms; and (3) circRNAs can be directly detected from cDNA sequences by designing multiple splice sequences.[26] At present, algorithms used for circRNA research include map-splice,[27] Circ Seq,[10] CIRI,[28] and Circ explorer.[29] The CIRI annotation-related algorithm can not only detect circRNAs transcribed from introns or intergenomic regions but also be applied to the sequencing data of annotated or unannotated eukaryotes. Since circRNAs lack a poly(A) structure, the common oligomeric dT enrichment method is ineffective. The Ribo-Zero kit, which is used to eliminate rRNA and RNase R to remove linear RNAs, can effectively enrich circRNAs.[20]

Functions

The functions of circRNAs are diverse and include adsorbing miRNAs as sponges, regulating selective splicing or transcription, interacting with RBPs, translating and deriving pseudogenes, and transporting substances and information. The functions of circRNAs are presented in Figure 2.

circRNAs Act as miRNA Sponges

circRNAs contain a common miRNA response element (MRE) that binds to miRNAs and prevents them from interacting with their target mRNAs.[30,31] The first proof of circRNAs acting as miRNA sponges was when cerebellar degeneration-related protein 1 antisense (CDR1as) RNA was determined to be related to miRNA to regulate its functions. CDR1as expression can reduce brain volume and hinder its development in the fetal development process of zebrafish embryos, and the injection of miR-7 can restore normal development, indicating that CDR1as may bind with miR-7.[31] circHIPK3 from exon 2 of the HIPK3 gene silenced HIPK3 mRNA and significantly inhibited the growth of human cells. Through luciferase screening, circHIPK3 silenced 9 miRNAs through 18 potential binding sites and directly specifically bound to miR-124 to inhibit its activity. However, bioinformatics analysis showed that circRNAs with a large number of miRNA-binding sites do not necessarily have a strong spongy effect, while other circRNAs confirmed this viewpoint.[32,33] Therefore, whether circRNAs act as miRNA sponges is a common phenomenon that remains to be explained.

circRNAs Regulate Selective Splicing or Transcription

circRNAs are involved in the regulation of variable splicing and transcription. Variable splicing is the process in which pre-mRNAs splice different mRNA isomers through different splicing methods (different splicing sites are selected), and circRNAs can be used for the regulation of variable splicing. For example, circMBL, produced from the second exon of the splice factor MBL (muscleblind), competes with pre-mRNA. circMbl and its side introns contain conserved MBL-binding sites, which are strongly and specifically inhibited by MBL. The regulation of the MBL level obviously influences the cyclization of circMbl, which is based on the MBL-binding sites in the intron sequence of the flanks.[6,7]

In addition, many other circRNAs contain translation initiation sites that potentially compete with their host gene pre-mRNA splices. This mode of regulation can balance the expression levels of circRNAs and the corresponding mRNAs. EIciRNAs can regulate protein production by regulating gene expression at the transcriptional or posttranscriptional level. For example, c-sirt7 can interact with the pol complex, leading to decreased expression of the related anchor protein repeat domain-52 or deacetylase-7. EIciRNAs, mostly localized in the nucleus, can interact with small ribosome U1 snRNP and then bind with pol to promote the transcription of parental genes.[18]

circRNAs Act as Sponges for RBPs

circRNAs, like some linear noncoding RNAs, may bind to RBPs to perform biological functions.[34,35] When combined with RBPs and ribonucleoprotein complexes, they act as sponges for RBPs, store them,[7] and then form complexes. EcircRNAs can bind stably and specifically to some protein molecules in cells. As a scaffold for binding RNA or DNA to complementary sequences, EcircRNAs provide an interaction platform for RBPs, RNA, and DNA. For example, CDR1as can bind to the miRNA action factor Ago2 protein to play a role in protein hydrolysis.[36] Du et al.[37] found that circ-foxo3 can inhibit cell cycle progression by binding to cyclin-dependent kinase 2 (CDK2) and the cyclin-dependent kinase inhibitor p21.

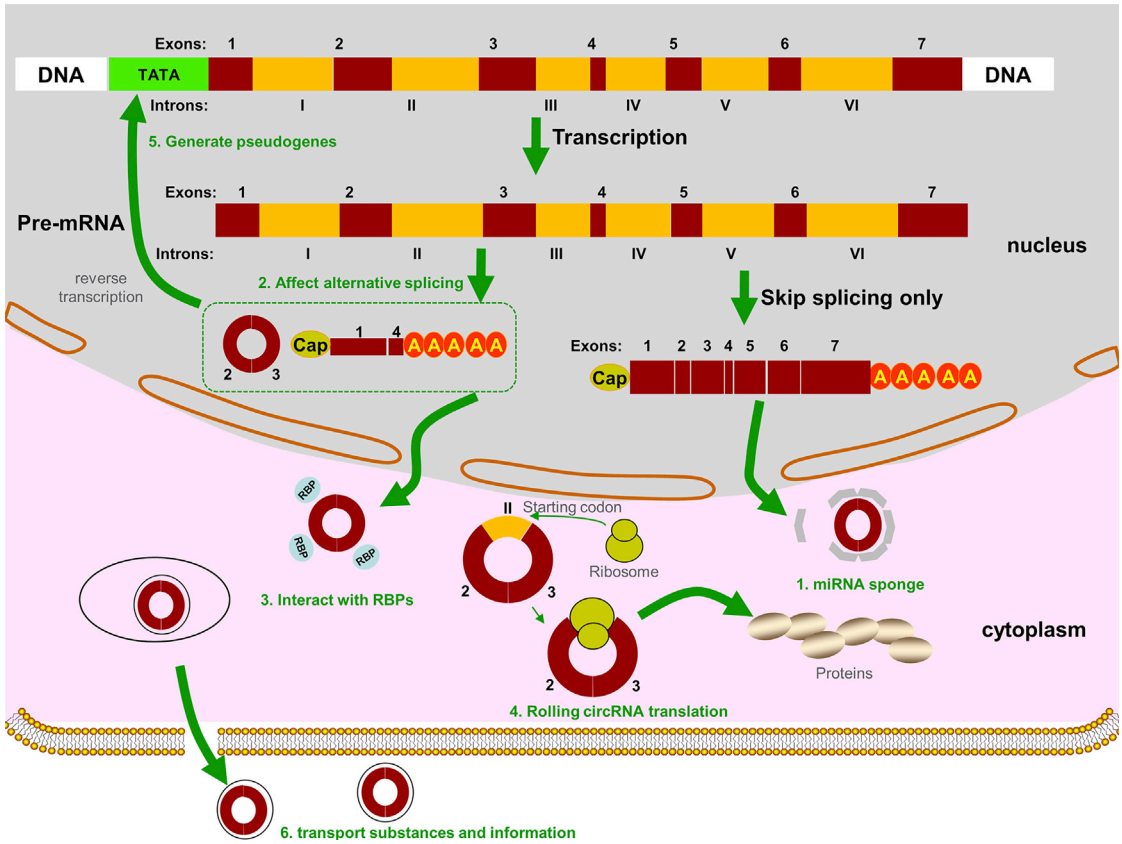

Figure 2. Functions of circRNAs

circRNAs can act as microRNA (miRNA) sponges, regulate selective splicing or transcription, use rolling translation, be combined with RBPs, and generate pseudogenes.

Abdelmohsen et al.[38] found that circ-PABPN1 could competitively inhibit the binding of the RBP HuR to poly(A)-binding protein nuclear 1 (PABPN1) mRNA, thus reducing the translation level of PABPN1 mRNA.

Variable splicing of RNA plays a major part in the occurrence of cancer, and cell proliferation is one of the main characteristics of cancer cells. By studying the role of RBP sponges on circRNAs, the biological function of circRNAs can be better understood, and new clues can be provided for the study of the role of circRNAs in tumorigenesis.

circRNAs Modulate Translation

As a species of noncoding RNAs, circRNAs basically do not encode proteins. However, if an internal ribosome entry point (IRES) is inserted upstream of the start codon of circRNAs, some circRNAs can also encode proteins that are functionally different from their linear transcripts. As previously shown *in vivo* and *in vitro*, an engineered circRNA including an IRES, eukaryotic ribosome 40S subunit, can bind to the circRNA on the IRES to start translation.[39,40] Similarly, in *Escherichia coli*, circRNAs with open reading frames of GFP can be transfected to express GFP.[39] Du et al.[41] proved for the first time that circRNAs are modified by m6A; that is, a methyl group is added to the sixth element of the base of the RNA molecule, and the modified circRNAs can be used for protein translation. Zhou et al.[42] also found that the m6A modification of circRNA showed cell specificity. Legnini et al.[43] reported that circ-ZNF609 is involved in the occurrence process of muscle and is directly used as the coding RNA to translate proteins. Yang et al.[44] found that circ-FBXW7 can translate a new protein that inhibits glioma, which is of great significance for understanding the function of circRNAs and for study on the mechanism of glioma.

circRNA-Derived Pseudogenes

Studies have indicated that stabilized circRNAs could form circRNA pseudogenes by retrotranscribing and integrating into the genome.[45,46] 33 high-confidence circRFWD2-derived pseudogenes, 9 low-confidence circRFWD2-derived pseudogenes, and 6 exon sequences outside of circRFWD2-containing pseudogenes were found by analyzing the circRFWD2 corresponding circle locus (exon 6-exon 2) in the mouse genome. Poly(A) is an important factor in RNA reverse transcription. 39 of the 42 circRFWD2-related pseudogenes do not contain poly(A) sequences, suggesting that some circRFWD2s can be retrotranscribed into cDNA in an unknown way. Therefore, the

Table 1. Cancer-Related circRNAs

Cancer	circRNA	Function	Signal Path	Effect
Gastric cancer[55,56]	circPVT1	miRNA sponge	miR-125	regulates cell proliferation; potential biomarker
	circ-LARP4	miRNA sponge	miR-424-5p	
	CDR1as	miRNA sponge	miR-7	anticancer
	circ-Foxo3	binds to protein	p21 and CDK2 proteins	regulates cell cycle progression
Human hepatocellular carcinoma (HCC)[52,57–61]	circZKSCAN1			inhibits proliferation, migration, and invasion
	CDR1as	miRNA sponge	miR-7	a risk factor
	hsa-circ-0001649			potential biomarker
	circRNA-100269	miRNA sponge	miR-630	inhibits proliferation
	hsa-circ-002059			potential biomarker
	circHIPK3	miRNA sponge		regulates proliferation
	hsa-circ-100338	miRNA sponge	miR-141-3p	associated with tumor metastasis and prognosis
	circ-MT01	miRNA sponge	miR-9	promotes p21 expression; suggests a poor prognosis
Colorectal cancer[62,63]	circ-CCDC66			promotes proliferation, migration, and invasion
	hsa-circ-0000069			promotes proliferation, migration, and invasion
	hsa-circ-001569	miRNA sponge	miR-145	a positive regulator of proliferation and invasion
	circ-ITCH	miRNA sponge	miR-7, miR-20 s	inhibits proliferation
	hsa-circ-001988			potential biomarker
Esophageal squamous cell carcinoma[64,65]	hsa-circ-0067934	miRNA sponge	miR-214, miR-98	promotes proliferation
	circ-ITCH	miRNA sponge	miR-17, miR-214	inhibits proliferation
	hsa-circ-001059	miRNA sponge		potential biological function in tumor radiotherapy resistance
Breast cancer[65,66]	CDR1as	miRNA sponge	miR-7	inhibits proliferation
	circ-ABCB10	miRNA sponge	miR-1271	promotes proliferation
	hsa-circ-0001785			potential biomarker
Lung cancer[65,67,68]	CDR1as	miRNA sponge	miR-7	inhibits proliferation
	circ-ITCH	miRNA sponge	miR-214	inhibits proliferation
	hsa-circ-0043256	miRNA sponge	miR-1252	inhibits proliferation; induces apoptosis
	circEA1			associated with cell differentiation and drug resistance
	circRNA-100876			potential biomarker
Bladder cancer[54,69,70]	circTCF25	miRNA sponge	miR-103a-3p, miR-107	promotes proliferation and migration
	circPTK2			promotes proliferation and migration; potential biomarker

interference of pseudogenes should be considered in future circRNA studies.[45]

circRNAs Transport Substances and Information

Exosomes are vesicular bodies secreted by a variety of cells, with diameters ranging from 40 to 100 nm. Their important characteristics are that they carry a variety of functional proteins from cells and mediate the exchange of substances and information between cells; therefore, they are called intercellular messengers. Recently, a large number of circRNAs were found in exosomes, indicating that circRNAs are also involved in the process of exosome function. circRNAs are abundant and stable in exosomes, and changes in circRNA expression also affect the occurrence and progression of disease.[47,48]

Mechanisms in Cancers

Most circRNAs have miRNA-binding sites that can be used as miRNA sponges to inhibit the regulation of miRNAs on downstream target genes by a large number of miRNAs in cancers. CDR1as, which can exert its sponging effect and bind to miR-7 in large quantities to inhibit the gene regulation of miR-7, thus indirectly achieving tumor inhibition, includes over 70 miR-7-binding sites. Previous studies have shown that the overexpression of miR-7 can significantly inhibit the proliferation and invasion of glioma, breast

cancer, gastric cancer, colorectal cancer, and other tumor cells.[49] circRNAs play an indirect regulatory role, mainly by acting as miRNA sponges. CDR1as/miR-7 is a relatively classical tumor-related circRNA-miRNA system.[30,36,50,51]

There are many other types of circRNAs with tumor-promoting effects, and the expression of these circRNAs in tumors is upregulated. circHIPK3 is a new tumor-related circRNA, discovered by Zheng et al.,[52] that is mainly found in the cytoplasm and plays a role in promoting cell proliferation by binding to miR-124. Xie et al.[53] found that has-circ-001569 could promote the proliferation and invasion of colorectal cancer cells by binding to miR-145 to upregulate the expression of E2F5, BAG4, and FMNL2, which are negatively regulated by miR-145. Zhong et al.[54] also found a significant upregulation of circTCF25 in bladder cancer, and they confirmed that the circTCF25-miR-103a-3p/miR-107-CDK6 pathway plays an important regulatory role in bladder cancer and the overexpression of circTCF25 can increase the proliferation and migration of cancer cells. Table 1 is a brief summary of some cancer-related circRNAs.

Conclusions

With the development of RNA-seq, an increasing number of studies have shown that circRNAs are significantly associated with many diseases, especially cancer. circRNAs are characterized by miRNA sponges that regulate transcription. Moreover, recent studies have found that circRNAs function in protein translation. Novel circRNA functions are constantly being described, and tumor development plays a significant role in their regulation. Studies of circRNA function in tumors can promote the understanding of disease progression and drive the cognition of clinical diseases, and it is the precondition of clinical application. The research value of circRNAs is reflected in their clinical applications. circRNAs can serve as biomarkers in the early diagnosis of tumors, and they can also be used as a sensitive indicator of the evaluation of curative effects and prognosis. Currently, the clinical applications of biomarkers, such as CEA, in a variety of digestive tract neoplasms, such as pancreatic cancer and colon cancer, are increasing. Therefore, it is difficult to avoid the lack of specificity in the diagnosis. circRNAs have tissue specificity and are better able to assist in making the diagnosis. Based on research on the regulatory mechanism of circRNAs, circRNAs can be used as anticancer targets as a new direction of tumor treatment. circRNAs related to the chemotherapy drug resistance of tumors will also become a new research topic.

AUTHOR CONTRIBUTIONS

J.W., X.Q., and L. Liu wrote and drafted the manuscript and figures. Jianming Yang, L. Lu, Z.Z., and S.M. collected the data. Z.W., Z.L., and W.Z. revised the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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