MINIREVIEWS

Circular RNAs in colorectal cancer: Possible roles in regulation of cancer cells

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Abstract

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world and the fourth principal cause of cancer deaths worldwide. Currently, there is a lack of low cost and noninvasive screening tests for CRC, becoming a serious health problem. In this context, a potential biomarker for the early detection of CRC has recently gained attention. Circular RNAs (circRNA), a re-discovered, abundant RNA specie, is a type of noncoding covalent closed RNAs formed from both exonic and intronic sequences. These circular molecules are widely expressed in cells, exceeding the abundance of the traditional linear mRNA transcript. They can regulate gene expression, acting as real sponges for miRNAs and also regulate alternative splicing or act as transcriptional factors and inclusive encoding for proteins. However, little is known about circRNA and its relationship with CRC. In this review, we focus on the biogenesis, function and role of these circRNAs in relation to CRC, including their potential as a new biomarker.

Key words: Circular RNA; Colorectal cancer; Gene regulation; CircRNA; Non-coding RNAs; Long non-coding RNA; Circularization

Core tip: Circular RNAs (circRNAs) are noncoding RNAs, characterized for its circularized shape. These circRNAs are abundant and might play important roles in cancer. In particular, they exhibit altered expression in colorectal cancer, and its activity as miRNA sponge might be involved in the control of cancer progression. Moreover, owing to their stability, could serve as diagnostic or predictive biomarkers for colorectal cancer.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world (1.4 million in 2012), and the fourth principal cause of cancer deaths worldwide (694000 deaths for both sexes)[1,2]. In terms of incidence, it represents almost 10% of global cancer diagnoses, and is the third most common cancer in males and second in females (746000 and 614000 respectively), with differences between developed and developing countries[2,3]. More diagnoses occur in developed regions, but less developed regions have higher death rates, reflecting a poorer rate of survival in these countries[1,3]. Sixty-five percent of the new cases occur in developed countries including the United States, Canada, Australia and Europe. In the United States in 2016, the American Cancer Society estimated over 134000 new diagnoses of CRC (95270 for colon, and 39220 for rectal cancer)[2,4]. Also in 2016, CRC is expected to cause over 49190 deaths in the United States[4]. Worldwide the mortality rates are lower in women than in men, except in the Caribbean. But this tendency seems less typical in countries with higher levels of human development[2]. In contrast, CRC incidence and mortality rates in Asia are continually increasing[5]. Besides Asia, in many regions of Europe and North America, the rates of CRC incidence and mortality have decreased for both men and women, due to new screening methods allowing for early diagnosis and treatment[4-6]. These screening tests can prevent the development of CRC, as some have the potential to detect polyps before they can progress into cancer. Not all polyps will progress into cancer tumors, but their removal can prevent the disease[6].

There are several factors that contribute to the development of CRC, some of these are malleable, while others, such as genetic factors, personal medical history, age, racial and ethnic background, or pre-existing conditions (inflammatory bowel disease, Lynch syndrome, MUTYH associated polyposis and others) are not[6]. CRC is primarily considered a "lifestyle" disease because its incidence is high in countries with a sedentary population and high-fat diets from animal sources[2]. An association between diet, obesity and carcinogenesis is a very important issue as several studies have shown that obesity promotes inflammatory processes, triggering a cascade of critical mechanisms mediated by proinflammatory cytokines and tumor necrosis factor-α (TNF-α), resulting in CRC development[7-9].

Three fundamental categories of genes have been identified in the carcinogenesis of CRC as follows: (1) *APC, DCC, TP53, SMAD2, SMAD4* and *p16INK4a* (tumor suppressor genes); (2) *K-ras* and *N-ras* (proto-oncogenes); and (3) *MMR* and *MUTYH* (DNA repair genes). However, little is known about the role of circular RNAs (circRNAs) in the development of CRC. CircRNAs are a class of small group of competing endogenous RNAs (ceRNAs), in the family of the non-coding RNAs (ncRNAs) that function as a class of long non-coding RNA (lncRNA)[10-12]. These molecules have captured the interest of many in the scientific and medical communities, because hundreds of human genes are expressed in circRNA form[13-15]. CircRNA has several functionalities, including the ability to rearrange the order of genomic information, provide protection from exonucleases, and establish constraints on RNA folding[16]. CircRNAs can function as templates for viroid and viral replication, as intermediates in RNA processing reactions, and regulators of transcription in *cis*. Additionally, it is speculated that circRNAs serve as epigenetic microRNA (miRNA) sponges, negatively regulating miRNAs and thus contributing substantially to the ceRNAs network through RNA-binding protein (RBP) sequestering agents, or nuclear transcriptional regulators, which are frequent in cancer[16-19]. There exists evidence that most circRNAs in the eukaryotic cell are stable, cytoplasmic, lacking the 3' poly(A) tail and 5' end cap, arising from pre-mRNA back-splicing exons (downstream 3' splice donors are covalently linked to upstream 5' splice acceptors) and host noncoding transcripts generated from protein coding genes[10,12,20-22]. Another study has found evidence that circular isoforms of several human transcripts, exhibiting the non-orderly fashion in the splice junctions, are expressed in similar concentrations to the normal linear isoform[23]. They hypothesized that scrambled exons are a sign of local genomic rearrangements in cancer, and realized this hypothesis by polymerase chain reaction (PCR), where the exon scrambling detected in leukemia patients was also found in normal primary human cell and Hela cells, suggesting that most exon scrambling found in tumor samples are consequences of an active splicing process both in normal and malignant human cells types[23]. This new type of ncRNA can be secreted by cells to the extracellular environment, and can be identified and measured through non-invasive methods, such as stool, blood and other body fluid sampling, suggesting that circRNAs could serve as an effective and cost effective screening test for CRC[15].

In this review, we discuss circRNAs, their biogenesis, possible functions, and implications in CRC.

LITERATURE SEARCH

A systematic literature search was performed of the PubMed database, and the keywords "circRNA", "circRNA" and "cancer", "circRNA" and "colorectal cancer" as well other synonyms for circRNAs, with no limitations. The inclusion criteria were as follows: (1) reviews about CRC incidence, statistics, and features of this disease before October 2016; (2) initial studies about circRNAs, its biogenesis, biological functions

and techniques used for its identification; (3) studies showing the presence of circRNA in human and other species, detected using different approaches either mathematical, biochemical, biological and technological or a combination; (4) investigations showing the expression of circRNA in different types of biological and cell samples; (5) studies associating circRNA with biological functions and try to explain its biochemical pathway; (6) investigations relating circRNA and CRC; including past, present and future of the disease; and (7) studies associating circRNA with colorectal human samples or cells and discussing its potential use as biomarker for early diagnosis of the disease.

PROPERTIES AND FUNCTIONS OF CIRCRNAS

In the large family of non-protein-coding RNAs, circRNAs are a novel small group of ceRNAs, that could function as a new class of lncRNAs[10-12]. CircRNA is most often composed of exonic sequences containing one or more exons, in contrast to linear RNA which is generally formed by a covalently closed continuous loop containing a 5'-3' phosphodiester bond and without polyadenylation[10,12,24,25]. This new type of RNA is now known to be expressed in all tissues, at generally low levels but highly represented in the eukaryotic transcriptome[19,26]. Several studies have shown that the circular form of circRNAs gives this molecule a special role in gene expression, but their biological functions have yet to be determined[17,26-29]. In 2013 it was reported that circRNAs are a common, abundant and potentially developmentally regulated component of the gene expression programs in diverse animal species[25]. Salzman *et al*[23] also demonstrated that circRNAs with scrambled exons compose a significant fraction of cellular RNA for many genes in cancer and normal human cells, suggesting also that the majority of scrambled exons detected in tumor samples were consequential of active splicing processes in both normal and malignant human cell types. Moreover, they subsequently reported that circRNA's relative abundance is regulated, in a gene and cell type specific manner, at levels comparable to those of canonical linear mRNA, and some of these circRNA species exhibited resistance to a 3'-5' exoribonuclease - RNase R[25]. Jeck *et al*[12] demonstrated in the same year, that there exist up to 25000 different circRNAs in human cells, and in some cases are even considerably more abundant (> 10-fold) than associated linear transcripts. Although most circRNAs are expressed at low levels, some have proved to be more abundant than their linear counterparts[25].

A distinctive property of circRNA is they differ structurally from other lncRNAs[19]. In order to create a linear RNA transcript with polarity 5' to 3', the eukaryotic cell uses spliceosomal machinery in conjunction with other biochemical process (5' capping and 3' polyadenylation) to create a canonical pre-mRNA,

removing introns and joint exons[10]. This splicing process is an ubiquitous feature of eukaryotic gene expression, and it is known that the Last Common Eukaryotic Ancestor was relatively intron-rich and had complex spliceosome machinery and splicing signals[21,22]. Conversely, most circRNAs in the eukaryotic cell are stable, cytoplasmic, lacking the 3' poly(A) tail and 5' end cap, come from pre-mRNA back-splicing exons and host noncoding transcripts generated from protein coding genes[10,12,20-22]. Guo *et al*[11] examined the subcellular localization of circRNA, focusing on the 514 circRNAs detected in the K562 whole-cell samples, and found these circRNAs predominantly in the poly(A)-depleted cytoplasmic samples. Jeck *et al*[12] demonstrated by FISH on Hs68 cells treated with Actinomycin D, against HIPK3; that circular forms of HIPK3 were preferentially localized in the cytoplasm as is consistent with prior studies of RNA circles. Apparently, endogenous circRNAs that are efficiently transported to the cytosol either undergo nuclear export or are released to the cytoplasm during mitosis, where they are extraordinarily stable[12,16,23,30]. This stability, or resistance, to debranching enzymes and RNA exonucleases[12] was also reported by Guo *et al*[11]; they found that most circRNAs that span < 5 exons are RNase R-resistant. Jeck *et al*[12] used a method described by Suzuki *et al*[31] in 2006, using *E. coli* RNase R, which degrades linear RNAs with short 3' tails regardless of secondary structure, leaving circRNAs unaffected[12]. Salzman *et al*[25] found that all the RNA species predicted to be circular are resistant to RNase R whereas all predicted linear sequences are highly sensitive to RNase R.

BIOGENESIS AND REGULATION OF CIRCRNAS

CircRNAs are the product of a back-splicing process also catalyzed by canonical spliceosomal machinery, and it has been reported that this process is modulated by *cis*-regulatory elements and *trans*-acting factors, but it is still unclear under which conditions the spliceosomal machinery achieve discrimination between canonical splicing or back-splicing to give rise to a circRNA[10,16,19,20,26,32]. Ashwal-Fluss *et al*[32] has provided evidence that circRNA are generated co-transcriptionally and their production rate is principally mediated by their flanking intronic sequences. They also demonstrated that canonical pre-mRNA splicing compete with circularization of exons, suggesting that a single pre-mRNA transcript can produce either a linear mRNA or a circular isoform (Figure 1A). Zhang *et al*[33] indicated that the back-splicing process is generally coupled with canonical splicing, but process happens first depends on the specific spliceosomal machinery and still is under investigation. It is important to know that different types of circRNA molecules are generated by distinct mechanisms, independent of if they arise from intronic or exonic sequences[16,26].
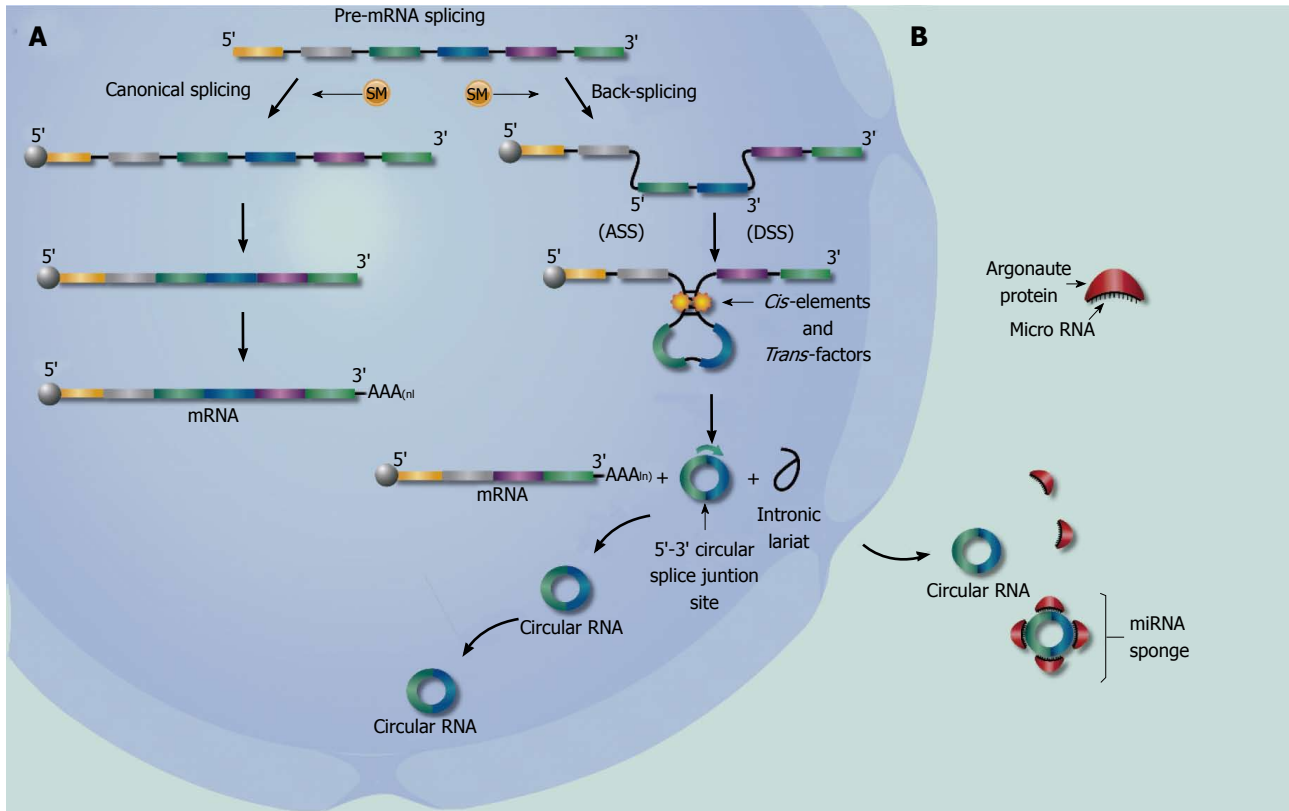
Figure 1 Biogenesis and function of circular RNAs. A: In the nucleus, a single pre-mRNA transcript can produce a linear mRNA and a circular isoform[32]. These two steps, canonical splicing and back-splicing are catalyzed by canonical spliceosomal machinery (Yellow circle, "SM"). In order to create a linear RNA transcript with polarity 5' to 3', the eukaryotic cell uses spliceosomal machinery SM together with other biochemical process (5' capping and 3' polyadenylation)[10]. Following, circular RNA is produced from a downstream 5'-DSS joined reversely to an upstream 3'-ASS, resulting in a covalently closed circRNA transcript with a 3', 5'-phosphodiester bond at the junction site (Figure 1)[16,22,26,33,34] and it seems to be modulated by *cis*-regulatory elements and *trans*-acting factors[10,16,19,20,26,32]. The final products of these processes are a linear mRNA, a circRNA, and an intron lariat; B: Endogenous circRNAs that are efficiently transported to the cytosol either undergo nuclear export or are released to the cytoplasm during mitosis, where are they are extraordinarily stable[12,16,23,30]. In the cytoplasm circRNAs form a class of post-transcriptional regulators, acting as highly stable epigenetic miRNA sponges competing with the endogenous RNA network (sequestering miRNAs from binding mRNA targets), directly affecting the expression of any related gene[17,18,46].

The process by which a pre-mRNA becomes a circRNA isoform begins with a start codon, and sometimes includes the canonical AUG of the associated linear transcript[12]. When the RNA Pol II recognizes the start codon, it transcribes the exonic circRNA; which is produced from a downstream 5'-DSS inversely joined to an upstream 3'-ASS, resulting in a covalently closed circRNA transcript with a 3', 5'-phosphodiester bond at the junction site[16,22,26,33,34], or through 2'-5' linkages formed by a branch-point nucleophilic attack during splicing[35]. The specificity of this process still being studied, but is interesting to note that introns flanking mammalian circRNAs are longer than average[23].

Two other mechanisms, direct back-splicing and lariat intermediate, have also been proposed as associated with circRNA formation from back-spliced exons[12,23]. This suggests that circRNA synthesis is evolutionarily dynamic, providing an alternative generation of circular transcripts[33]. Furthermore, some exons that generally are flanked by longer introns (upstream or downstream), result in a less efficient splicing process. Inverted repeat elements, such as ALU, can facilitate RNA circularization in humans[12,33,34]. Zhang *et al*[33]

identified the phenomenon "Alternative Circularization" (AC), in which a number of multiple exon circularization events can be produced from a single gene loci. This novel process of splicing leads to formation of multiple circRNAs with different expression levels, and it has been associated with diverse biologic processes[12,36,37]. In some events, AC leads to circRNAs retaining introns, adding an additional layer of complexity to the circRNA transcriptome[22,25,26]. It has been suggested that the formation of these isoforms is derived from exon-skipping events. In these events, an exon-containing lariat is formed, which can then be internally spliced to create an exon circle[12,38].

The back-splicing process appears to be regulated by the ratio of circular to linear transcripts, and the relative abundance of differentially spliced circular isoforms is cell-type specific[22,25]. In general, the efficiency of the back-splicing process seems to be much lower than canonical splicing, in steady-state levels of circRNAs; but this efficiency is dependent on the presence of canonical splice sites flanking the exons[20,26,32,33]. Besides, flanking intronic sequences are the main factor determining circularization efficiency of a given exon, and this

efficiency is related to the size of the intronic sequence. For example; circRNA flanked by longer introns are less efficiently spliced than other circRNA flanked by smaller introns[32]. Moreover, this circularization appears to have a negative effect on the splicing efficiency in the linear isoform and therefore on gene expression[32]. To overcome this inconvenience, most circRNAs have regulatory elements which reside in the flanking introns of circularized exons to continue with the back-splicing process and facilitate circRNA formation[10,26]. It has been shown that RBPs can regulate and promote circularization, after being recognized by regulatory elements such as short *cis* elements[26,32].

Another way to facilitate the processing of circRNAs is by RNA base pairing. Complementary short repeat elements, such as inverted ALU repeats, in the flanking introns are used to circularize some intervening exons. This mechanism suggests that the promotion of back-splicing requires for exons to collaborate with intronic repeats and join the back splice sites, thereby facilitating catalysis through base pairing[12,39]. However, in the case of base pairing, the simple presence of these inverted repeats does not necessarily mean that a circRNA will be produced with similar efficiency in all cell types[39]. It is suggested that RBPs, base pairing, and any splicing factor (*cis* regulatory elements and *trans* factors), might determine the balance between canonical RNA splicing and back-splicing processes, but whether circRNA is produced co-transcriptionally or post-transcriptionally is still to be determined.

FUNCTIONS OF CIRCULAR RNAS

Little is known about circRNA biology, and a large number of possible functions have been attributed to this circular isoform. In human cells[40], another class of nuclear circRNAs has been identified by Gardner *et al*[41]: Circular intronic RNAs (ciRNAs) or stable intronic sequences RNA (sisRNA). In human cells (HeLa and human embryonic stem cells hESCs-H9), exon-intron circRNAs were reported, finding that these circRNAs, associated with the elongation Pol II machinery, act as positive regulators of Pol II transcription, suggesting a *cis*-regulatory role of noncoding intronic products in the efficient transcription of their parent coding genes[42]. CiRNAs do not function only as microRNA sponges, but, some abundant ciRNAs may be able to regulate gene expression in *trans* under certain circumstances.

A wide variety of studies have demonstrated that abnormal expressions of circRNAs are closely associated with various diseases including CRC[43-45]. It has been speculated that circRNAs form a class of post-transcriptional regulators, acting as epigenetic, highly stable miRNA sponges to compete with the endogenous RNA network, directly affecting the expression of any related gene (Figure 1B)[17,18,46]. CircRNA could be sequestering specific miRNA complexes and releasing them after cleavage[17]. The miRNA sponges ciRS-7 and miR-7, highly expressed in brain, interact to form a miR-7 inhibitor/sponge complex. It is possible that the competition between ciRS-7 and miR-7 effects oncogenesis[18,47]. Similarly, the testis-specific circRNA (*Sry*) regulates activity of miR-138, a tumor suppressor[17,48]. Therefore, ciRS-7 and *Sry* complex with miR-7 and miR-138, respectively, to form microRNA sponges blocking functionality, and reducing the invasiveness, metastasis and proliferation of several cancers[47].

CRC shows a negative correlation between global reduction of circRNA and cell proliferation, suggesting that the back-splice machinery responsible for RNA circularization, is dysfunctional in tumor cells[46]. Recently, Xie *et al*[49] found an association between miR-145 and hsa_circ_001569 and the regulation of CRC progression.

Another circRNA, cir-ITCH acts as a miRNA sponge with an inhibitory effect on esophageal squamous cell carcinoma. Cir-ITCH stimulates ITCH levels, provoking an ubiquitin-mediated Dvl2 degradation, and inhibition of the canonical Wnt/β-catenin pathway, finally, contributing to cancer tumor advancement[50]. The expression of hsa_circ_001988 is downregulated in CRC, and contributes to differentiation and perineural invasion[51]. A study in breast carcinoma cells (MDA-MB-231)[52] shows the possible functions of circ-Foxo3 (hsa_circRNA_104170), which binds to eight miRNAs (miR-22, miR-136*, miR-138, miR-149*, miR-433, miR-762, miR-3614-5p and miR-3622b-5p), as well as Foxo 3 Protein (Foxo3P) and Foxo3 mRNA. Circ-Foxo3 has a sponging effect on these miRNAs, promoting Foxo3 mRNA translation, suppressing both tumor growth and cancer cell proliferation[52].

CIRCRNAS IN CRC

Recent reviews have reported that in CRC cell lines and CRC tissues, a global reduction of circRNA abundance is observed, in comparison to healthy tissue, therefore allowing for the proliferation of CRC cells[53,54].

CircRNAs were associated for the first time with CRC in transcripts of DCC (Deleted in CRC)[30]. In 2015, Bachmayr-Heyda *et al*[46] reported a global reduction of circRNA abundance in CRC cell lines and tumor samples, as compared to normal mucosa in patients with CRC. Using RNA-seq and the algorithm described by Memczak *et al*[21] and found by RT-qPCR, 39 circRNAs differentially expressed in the normal colon mucosa and CRC samples, in which 11 of the circRNAs were upregulated and 28 were downregulated. Interestingly, the Bachmayr's group found that the expression of circRNAs was reduced in tumor samples (circRNA expression 27.8%) compared to normal colon mucosa samples (circRNA expression 78.1%). Similar evidence was found in 11 CRC cell lines, with even wider gaps in expression ratios[46]. In order to validate reduced circRNA expression in CRC compared to normal mucosa samples, they enriched circRNAs through RNase R digestion and subsequent deep sequencing, reporting 21653 distinct

Name	Location	Length [nt]	Tissue/Cell line	Dysregulation	Gene	miRNA associated	Ref.
DCC	chr18q21.2	253, 948	HCT116	None	*DCC*	None	[30]
circ0817	chr11	653	CRC tissue and CRC cell lines	Downregulated	*CUL5*	None	[46]
circ3204	chr15	706	"	Downregulated	*USP3*	None	[46]
circ6229	chr14	629	"	Downregulated	*METTL3*	None	[46]
circ7374	chr17	288	"	Upregulated	*TNS4*	None	[46]
circ7780	chr7	317	"	Downregulated	--	None	[46]
circ_001988	chr4q3	-	CRC tissue	Downregulated	*CDR1as*	miRNA-7	[21,51]
circ_001569	chr16q13.1	-	CRC tissue	Upregulated	*ABCC1*	miRNA-145	[49]

CRC: Colorectal cancer.

back spliced junctions. This method revealed that it is impossible to ensure the detection of all circRNA, and that the actual number of circRNA species is much higher. This was also corroborated in other cell lines in a non-cancerous neo-proliferative disease, showing a similar negative correlation as the circRNA index and proliferation observed in colon tissues and cell lines[46]. Finally, the authors hypothesized that the back-splice machinery responsible for RNA circularization is dysfunctional in tumor cells due to an increased degradation by oncomiRNAs[15,46]. Recently, Wang *et al*[51] reported similar findings concerning the correlation of hsa_circ_001988 abundance and CRC in tumor tissue and adjacent normal mucosa from 62 CRC patients. By RT-qPCR they verified the presence of hsa_circ_001988 in these tissues, and it was found to be significantly downregulated in tumor tissue compared to healthy samples. The expression level of hsa_circ_001988 was significantly related to differentiation ($P < 0.05$) and perineural invasion ($P < 0.05$). Perineural invasion is a predictor of outcome in CRC and negatively associated with survival time and local recurrence in CRC patients. They concluded that hsa_circ_001988 may play a profound role in differentiation and perineural invasion and could be a potential target to regulate cytological behaviors[51]. Recently Xie *et al*[49] provided information about an up-regulated circRNA (circ_001569) complexing with the tumor suppressor miR-145. MiR-145 has been related with patient survival after CRC diagnosis[55-57]. They explored the expression pattern of circ_001569 by real-time PCR in 30-paired samples of CRC patients, and found expression of circ_001569 was significantly higher in the CRC tissues and found correlation with aggressive characteristics of CRC, including distant metastasis and poor differentiation. Then to detect the function of circ_001569 in the progression and invasion of CRC, they proceeded to over-express and silence the circular isoform in four CRC cell lines (over-expressed in SW480 and HCT116 and silenced in SW620 and LOVO). Accordingly, over-expression increased proliferative and invasive ability in circ_001569 expressing cells, while a sharp reduction in proliferation and invasion rates was shown in circ_001569 silenced cells. In addition, they found the level of miR-145 was significantly lower in CRC tissues[49]. Furthermore, using the bioinformatic algorithms (TargetScan, Pictar and miRANDA), along with Luciferase action, RT-qPCR and Western Blotting assays, they found that circ_001569 increased the protein levels of E2F5, BAG4 and FMNL2 in SW480 and HCT116 cells, and knockdown of the circRNA in SW620 and LOVO cells had the opposite effect. Here, they provided evidence showing tumor promoting functions (proliferation and invasion) of circ_001569 in CRC cells, directly inhibiting the regulatory activity of miR-145, and subsequently up-regulating its protein targets E2F5, BAG4 and FMNL2. Finally, they concluded that this interaction between miR-145 and hsa_circ_001569 in regulating CRC progression may provide new insights and therapeutic strategies for CRC prevention and treatment[49].

CircRNAs that have been related with CRC are shown in Table 1.

CONCLUSION

The information reviewed is the beginning of a better understanding of mechanisms and functions of this recently re-discovered circular isoform. CircRNAs can act as competing endogenous RNAs or miRNA sponges, regulating alternative splicing or transcription, modulating the expression of parental gene, managing local concentration of RBPs and RNAs through RNA transport. In cancer these molecules complex with miRNAs as inhibitor/sponge, with profound effects on oncogenesis and regulation of cancer pathways. It is possible that more important functions will be associated to this circular transcript in the near future. Still, the molecular mechanisms concerning the positive or negative relationship between circRNAs and miRNAs in CRC are poorly understood.

The search for a suitable circRNA biomarker for CRC is an important topic requiring further study. It is probable that the principal role of circRNAs in the progression of cancer is the modulation of the gene expression of oncogenes and or tumor suppressor genes, through the control of miRNA targets.

Summarizing, we conclude that microRNAs expression levels are regulated by circRNAs in a cell-type specific way, playing an important role in oncogenesis and the malignant behavior of cancer. Nevertheless, the function between circRNA and cancer, as well as the regulatory mechanisms is still elusive and needs to be further explored. Someday this circular isoform might become an important biomarker with a potential use in the diagnosis, prognosis and therapy of CRC.

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