Neoplasma 2022; 69(3): 516–526

doi:10.4149/neo_2021_210718N986

Exosomal circRNAs as novel potential biomarkers for colorectal adenoma

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Received July 18, 2021 / Accepted August 25, 2021

Growing evidences have revealed that exosomal miRNAs, lncRNAs, and circRNAs play a pleiotropic role in tumor biology. Cell-cell communication mediated by exosomes has been considered to be a key factor in the malignant progression of colorectal cancer. However, the importance of exosome-derived circRNAs in the biological function and clinical significance of colorectal adenoma remains elusive. In this study, we aimed to identify altered circRNA expression profiles in exosomes isolated from plasma of patients with colorectal adenoma using high-throughput sequencing. Exosomes were confirmed by western blotting, transmission electron microscopy, and NanoSight assay. The sequencing data indicated that there are 413 differentially expressed circRNAs including 112 upregulated and 301 downregulated circRNAs in colorectal adenoma patients compared with controls. GO analysis and the circRNA-miRNA-mRNA network were performed to predict the potential function of circRNAs, and demonstrate the putative mechanisms in colorectal adenoma. Collectively, our findings revealed that plasma exosomal circRNAs may be a potential noninvasive biomarker for the detection of colorectal adenoma, and provided new insights into colorectal adenoma-carcinoma sequence.

Key words: circRNAs, exosome, colorectal adenoma, plasma, high-throughput sequencing

Colorectal cancer (CRC) is the most prevalent cancer and the second leading cause of cancer-related death [1]. The majority of colorectal adenomas are thought to be the precursor lesions to CRC. Over time, colorectal adenomas increase in size, develop increasingly dysplastic characteristics, and can eventually acquire invasive potential. The process of sequential alterations takes about 8–15 years [2]. The stepwise progression varies depending on the different molecular mechanisms. The screening programs is gradually popularization, especially endoscopic examination, many high-risk adenomas can be found at the time. Colonoscopy is the main screening method of the colon and rectum, which provides visualization of the entire large intestine, and can be used for localization, biopsy, and resection of a potential precancerous lesion [3]. The disadvantage of this method is an invasive operation, which is uncomfortable, and bowel preparation required drinking a lot of laxatives is also unpleasant. If it is an experienced expert to do the inspection, the cost will be higher [4]. There are other examinations, methods, including stool testing, blood testing, radiological examination, and so on [5, 6]. Therefore, more affordable, minimally invasive, and non-invasive approaches are much needed to improve the overall sensitivity and patient compliance for colorectal adenoma screening.

Circular RNAs (circRNAs) are newly discovered non-coding RNAs, which have a closed circular structure without 5'caps and 3'poly tails [7–10]. The unique structure of circRNAs can resist the degradation of RNase R, making it more stable than linear parent genes [11-13]. CircRNAs have features that are ideal properties of biomarkers, including conservation, abundance, and stability in plasma, saliva, and urine [14]. Recently, there have been reports that circRNAs are abnormally expressed in tumor tissues and cells, and participate in the process of malignant progression [15–20]. CircRNAs have the capacity to serve as a microRNAs (miRNAs) sponge and block the inhibition of miRNAs on their target genes, which may be implicated in cancer malignant behavior [21–23].

Exosomes are nanoscale vesicles secreted by cells, which have attracted more and more attention in the past decades. Previous reports have shown that exosomes contain various biomolecules, such as proteins [24], lipids [25], and nucleic acids [26]. Exosomes can help to treat different types of cancer, inhibit the local and distant spread of the tumor, and have the potential of early detection of cancer and inhibition of drug resistance [27]. In addition, exosomes are always abnormally expressed in malignant progression and can be found in the early stage of cancer. It is well documented

that exosomal RNAs could be promising clinical biomarkers for various cancers [28, 29]. Furthermore, tumor-derived exosomes play a role in signal transduction of intercellular communication [30–32]. In the process of malignant progression, tumor cells release exosomes and regulate the tumor microenvironment. In addition, tumor cells secrete exosomes, which change the composition of lung, liver, and other distal organs, and contribute to the distant metastasis of the tumor. Because the secreted or excreted exosomes, such as saliva, ascites, and cervicovaginal lavage fluid, can be evaluated noninvasively or minimally invasive. If exosomal RNAs can be used as a diagnostic, prognostic, or predictive biomarker, doctors can obtain samples from patients for diagnosis and prognosis through relatively noninvasive methods in the future. In the recent five years, exosomal RNAs as a clinical biomarker have achieved some promising results in various types of tumors.

However, the diagnostic value of plasma circRNAs for colorectal adenomas or early tumors is still unclear. In this study, we investigate the circRNAs' expression profiles in plasma exosomes from colorectal adenoma and healthy controls. We further explored the possible molecular mechanism associated with the differentially expressed circRNAs by GO analysis. Our work provides novel insight into novel potential biomarkers in the detection of colorectal adenomas or early tumors.

Patients and methods

Patients and plasma specimens. All samples used in this study were from our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China). Diagnosis of colorectal adenoma was confirmed by histopathological analysis. None of the patients received preoperative chemotherapy or radiotherapy. EDTA blood tubes were used to collected plasma samples. Sample collection, use, and storage procedures used in this study were approved by the Ethics Committee of Zhejiang University.

Plasma exosome isolation. Exosomes were separated by the ExoQuick exosome precipitation kit (System Bioscience, Palo Alto, CA, USA), according to the manufacturer's directions. To be brief, the pelleted exosomes were resuspended in 1× phosphate-buffered saline (PBS) and their concentration was estimated by the Bradford assay. Exosomes were stored at –80 °C prior to further use.

Western blotting. The expression of the exosomal markers (CD81 and CD63) was analyzed by western blotting. Protein concentration was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins were denatured by heating at 90 °C for 10 min in 4× nuPAGE LDs sample buffer (Life Technologies, Carlsbad, CA, USA). Equal amounts of protein from each sample were separated by 6–18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Life Technologies). Then, the proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membrane was incubated overnight at 4 °C with the primary anti-CD81 antibody (1:1000, ab109201, Abcam, Cambridge, MA, USA) and anti-CD63 (1:1000, ab216130, Abcam, Cambridge, MA, USA). After washing three times with Tris-buffered saline with 0.05% Tween-20 for 10 min each, the signal was detected with the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Transmission electron microscopy. Exosomes were re-suspended in 200 μl PBS and then this solution was transferred to a carbon-coated Cu grid (ProSciTech, Kirwan, QLD, Australia) and incubated at room temperature for 5 minutes. Exosomes were negatively stained with aqueous uranyl acetate of 2% for 2 minutes and washed three times in PBS. After drying, the morphologies of isolated exosomes were observed by transmission electron microscopy (TEM; JEOL JEM-1010, Japan) at room temperature.

RNA isolation, library construction, and RNA-seq analysis. High throughput sequencing was carried out by Cloud-Seq Biotech (Shanghai, China). We used the Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA, USA) to remove the rRNA from total RNA following the manufacturer's instructions. The CircRNA Enrichment Kit (Cloud-seq, USA) was used to enrich the circRNAs. RNA library was constructed with TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA), and the quality and quantity of the library were controlled by the BioAnalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA). After libraries were transformed into single-stranded DNA molecules, the product was captured by Illumina and sequenced for 150 cycles on Illumina HiSeqTM 4000 Sequencer (Illumina, San Diego, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression of the candidate circRNAs was validated by qRT-PCR. Total RNA concentration was quantified by NanoDrop spectrophotometer (Thermo Fisher Scientific) at 260 nm. Template complementary DNA (cDNA) was synthesized by PrimeScript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. The resultant cDNA was then used as a template for the real-time PCR amplification of the SYBR Premix Ex Taq II (Takara, Japan) operated on the Bio-Rad QX100 Droplet Digital PCR system (USA). Relative RNA amount was calculated by the $2^{\Delta\Delta Ct}$ method with the normalization to GAPDH. The primers were as follows: GAPDH-Forward: GGGAGCCAAAAGGGTCATCA, and Reverse: TGATGGCATGGACTGTGGTC; hsa_circ_0004075-Forward: GGAACAGACTCCACCCAAACAT, and Reverse: TCATTGTCATCGTCATCACTGTC.

Statistical analysis. All data were presented as the mean ± SD. Statistical analysis was processed by the Statistical Program for Social Sciences (SPSS) 22.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The unpaired Student t-test was used to analyze qRT-PCR results between the case group

and the control group. The value of $p<0.05$ was regarded as a statistically significant difference.

Results

Characterization of plasma exosomes. To determine the characteristics of the exosomes, we performed TEM, western blot, and NTA. Plasma exosomes from colorectal adenomas patients and healthy controls expressed abundant exosomal markers CD63 and CD81, as shown by western blot (Figure 1A). TEM showed that exosomes exhibited round-shaped morphologies with mean diameters around 76.89 nm (Figure 1B). The exosomes size distribution was measured by NTA. The NTA results illustrated that the concentration was 2.4E+7 particles/nm and the mean size was 76.89 nm (Figure 1C). The above analysis indicated that the plasma-derived particles we isolated were exosomes.

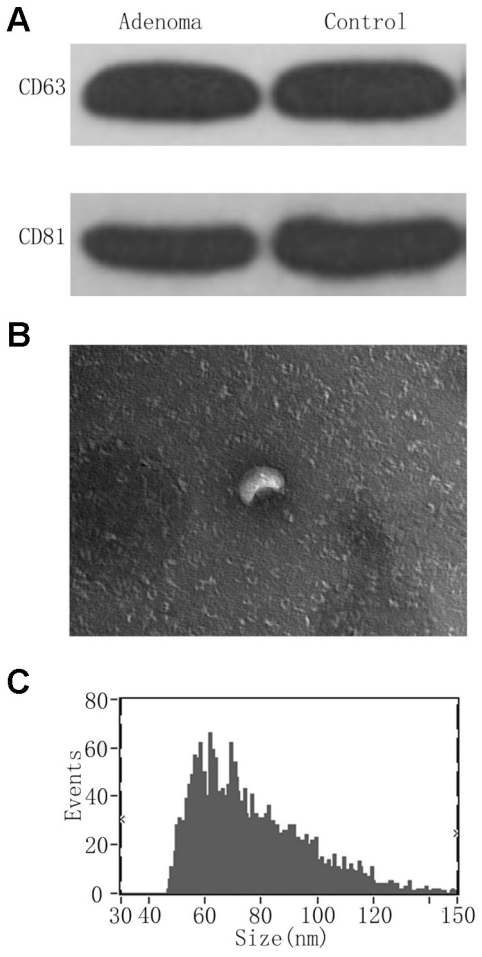

Figure 1. Identification of exosomes isolated from plasma. A) Exosomal markers (CD63, CD81) in representative plasma exosomes were determined by western blot. B) The morphology and size of plasma exosomes detected by TEM. C) The size and concentration of serum exosomes measured by NTA. NTA, Nanoparticle Tracking Analysis; TEM, transmission electron microscopy.

Differential analysis of exosomal circRNAs. The circRNAs profiles in exosomes from plasma were analyzed by RNA deep sequencing. The circRNA expression profile of the colorectal adenomas and controls is shown in a hierarchical clustering heatmap (Figure 2A). A circRNA candidate was considered to be significantly different according to statistical criteria of fold change ≥2.0 and p-value ≤0.05. A scatter plot (Figure 2B) and a volcano plot (Figure 2C) of all the differentially expressed circRNAs exhibited the distinguishable circRNA expression profiles between colorectal adenomas patients and healthy controls. In total, among the 35,191 identified circRNAs, 112 were significantly upregulated and 301 were significantly downregulated in colorectal adenomas compared with controls (Figure 2D).

Exosomal circRNA profiling in the plasma. To further uncover the characteristics of the differentially expressed circRNAs, we classified them according to their different positions on chromosomes. Our results revealed that the circRNAs are situated at all of the human chromosomes (Figure 3A). Moreover, among these circRNAs, 11.62% were intronic circRNAs, 66.59% were exonic circRNAs, 5.08% were antisense circRNAs, and 16.71% of the circRNAs were of other sources (Figure 3B). Among the 112 upregulated circRNAs, 35 were confirmed as novel circRNAs, 77 are listed in the circBase, but in the downregulated circRNAs, 184 were identified as novel, while 117 circRNAs had been collected in databases (Figure 3C). Moreover, the length of these circRNAs was investigated; the majority of the differentially expressed circRNAs were <2,000 nucleotides (nt) in length (Figure 3D). The top 10 significantly upregulated or downregulated circRNAs in exosomes were listed in Table 1.

Functional analysis of the differentially expressed circRNAs. GO and KEGG analysis were applied on their parental genes to predict their potential functions in our study. GO annotation analysis elucidates the enrichment terms of biological processes (BP), cellular components (CC), and molecular functions (MF), thus further revealing the biological functions of upregulated circRNAs. The most highly enriched GO terms in biological processes were "organelle organization" and "protein sumoylation" (Figure 4A). The most highly correlated cellular component was primarily involved with "intracellular" and "intracellular organelle" (Figure 4B). Based on molecular functions, "DNA binding" and "binding" are closely related to these circRNAs (Figure 4C). KEGG pathway analysis (Figure 4D) suggested that the upregulated circRNAs were involved in several biological pathways. Among these pathways, the GnRH signaling pathway and MARK signaling pathway are the most prominent, which may be related to the molecular mechanism of pathogenesis.

Validation of exosomal circRNAs expression and circRNA-miRNA-mRNA network. To further validate exosomal circRNA-seq results, the exosomes isolated from 12 pairs of samples (12 colorectal adenomas and 12 controls), were used to extract the RNA for qRT-PCR. The

levels of hsa_circ_0004075 in colorectal adenomas patients were significantly higher than the controls (Figure 5A), which was consistent with the sequencing results and confirmed the elevated expression of hsa_circ_0004075 in the plasma exosomes of the patients. It was reported that circRNAs could serve as miRNAs sponges to regulate gene expression [33]. To further unveil the putative miRNAs in colorectal adenomas, target miRNAs and mRNAs of the

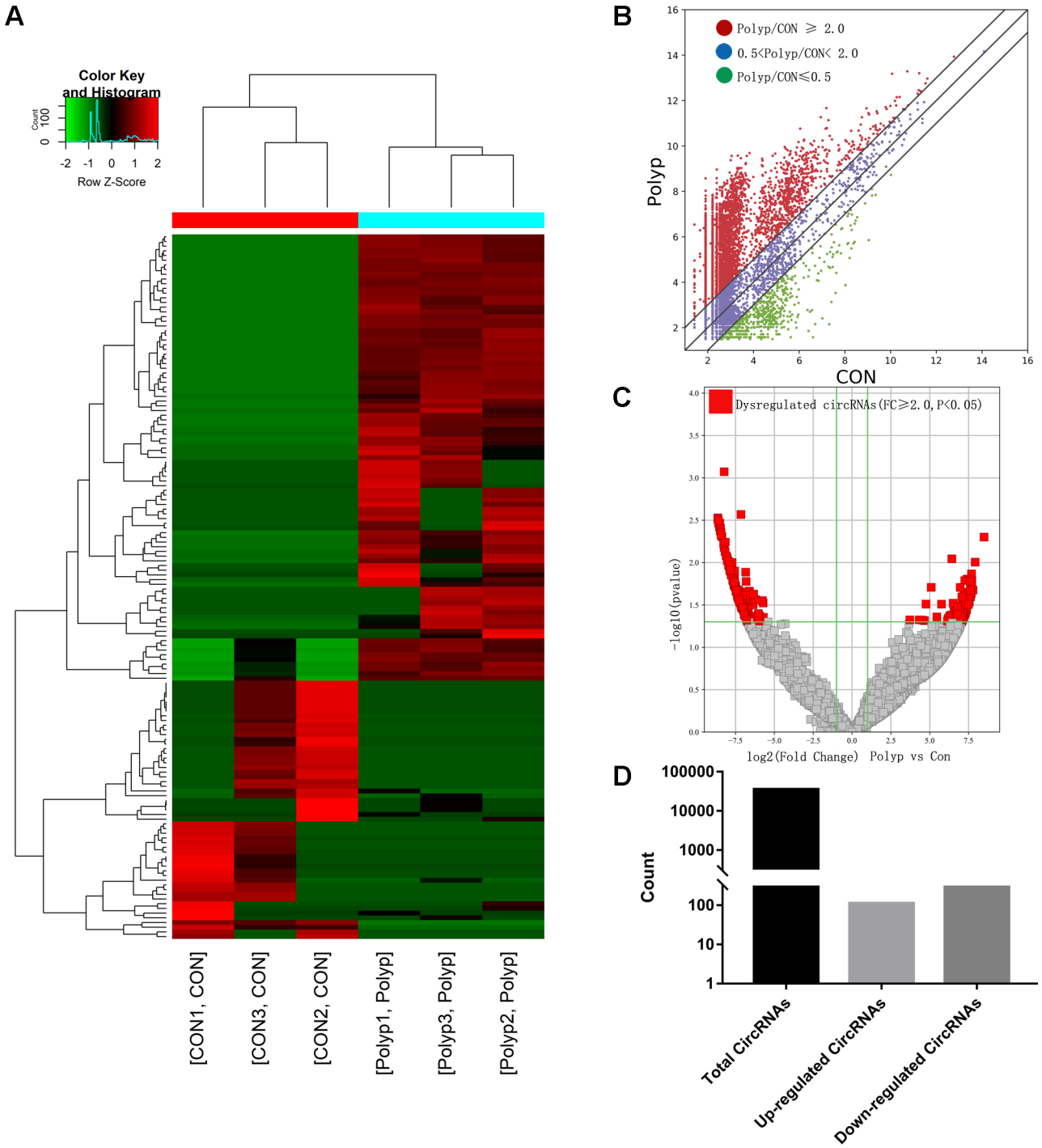

Figure 2. Differential analysis of exosomal circRNAs between colorectal adenomas patients and control individuals. A) Hierarchical clustering showed differences in circRNAs expression profiles between the two groups. B) The changes of circRNAs expression were visualized by scatter plot. C) The volcano plot revealed expression profiling between the two groups. D) The number of upregulated and downregulated circRNAs was displayed.

Table 1. The top 10 exosomal circRNAs with downregulated and upregulated expression.

CircRNA ID	Log FC	p-value	CircBase ID	Gene name	Catalog
chr2:121112155-121112311+	–8.636884095	0.00307173			intergenic
chr6:136977445-137041727-	–8.636884095	0.00307173	hsa_circ_0077977	MAP3K5	exonic
chr7:7622749-7629194+	–8.634455762	0.002968638		MIOS	exonic
chr6:84922675-84925640-	–8.59841348	0.003196802	hsa_circ_0077228	CEP162	exonic
chr19:14023136-14024451+	–8.585905697	0.0034144		CC2D1A	exonic
chr1:57766077-57766273+	–8.533072267	0.003798969		DAB1	antisense
chr1:235602084-235606226+	–8.508983858	0.003418473	hsa_circ_0112516	TBCE	exonic
chrM:13979-15632-	–8.478243893	0.004231869		JA760602	sense overlapping
chr1:28818162-28819603+	–8.462067206	0.003950038	hsa_circ_0011143	PHACTR4	exonic
chr17:2726836-2740135+	–8.432797171	0.00442625		RAP1GAP2	intronic
chr11:47774468-47776216-	8.50022658	0.005000554	hsa_circ_0004075	FNBP4	exonic
chr12:110922883-110925748+	7.930254881	0.009884176	hsa_circ_0004516	FAM216A	exonic
chr12:123497160-123498589-	7.786277233	0.021099941	hsa_circ_0029170	PITPNM2	exonic
chr10:20436713-20466338+	7.717343152	0.016165229	hsa_circ_0000224	PLXDC2	exonic
chr17:60629663-60642498+	7.707548797	0.018901116	hsa_circ_0008371	TLK2	exonic
chr22:38917613-38964294-	7.69510056	0.013608844	hsa_circ_0001231	DMC1	exonic
chr4:83852096-83867627-	7.679225451	0.023261962	hsa_circ_0127102	LIN54	exonic
chr18:67540380-67614669-	7.666702322	0.018706079		CD226	exonic
chr5:94275788-94353188-	7.632999255	0.025364699	hsa_circ_0130059	MCTP1	exonic
chr10:11978544-11994248-	7.620493417	0.021040435	hsa_circ_0017705	UPF2	exonic

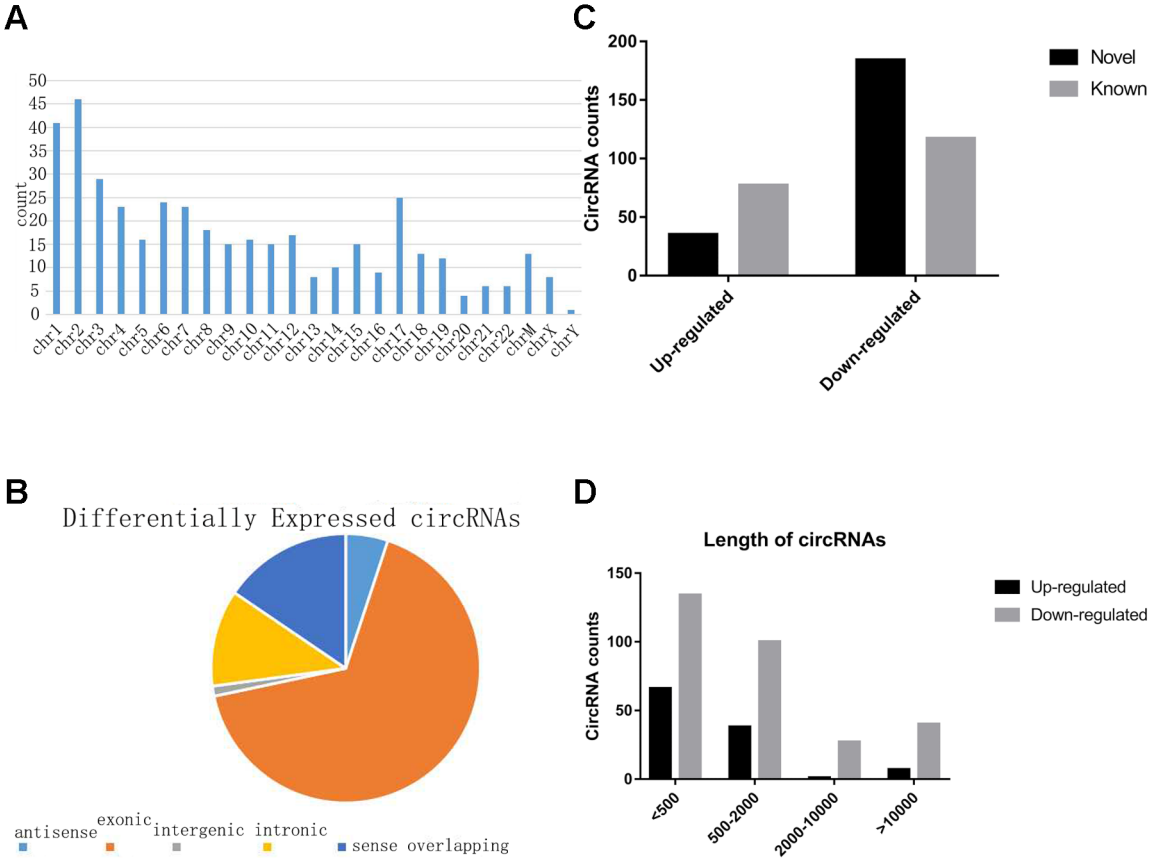

Figure 3. Profiling of circular RNAs in human serum exosomes. A) The distributions of the differentially expressed circRNAs in human chromosomes. B) Classification of circRNAs based on the genomic origin. C) Among the differentially expressed circRNAs, the novel and known circRNAs were uncovered. D) Length distribution of the circRNAs was unveiled.

hsa_circ_0004075 were predicted by miRanda and Target Scan. And the top 5 putative binding miRNAs are hsa-miR-6880-5p, hsa-miR-6511b-5p, hsa-miR-6851-5p, hsa-miR-6740-5p, and hsa-miR-5004-3p. A circRNA-miRNA-target gene network for hsa_circ_0004075 was built by Cytoscape (Figure 5B).

Discussion

Colorectal cancer is still a very common cancer worldwide because it is the third most frequently diagnosed cancer in men and women [34, 35]. However, the underlying causes and molecular mechanisms of CRC remain to be understood. The occurrence and progression of CRC is a comprehensive process from benign adenoma to cancer. Alterations of key regulatory genes in the adenoma-carcinoma sequence mark the transition from normal to neoplasm. It was also found that adenoma removal significantly reduced the risk of death from colorectal cancer, as compared with that in the general population [36].

Early detection and complete resection of precursor lesions could effectively halt the polyp carcinogenesis [37, 38]. At present, colonoscopy is the widely used method of colorectal examination. Although it is considered the gold standard for diagnosis and treatment of adenoma, colonoscopy has its limitations and is user-dependent, such as diet restriction, bowel preparation, and well-trained examination. The polyp miss rate as determined by colonoscopy

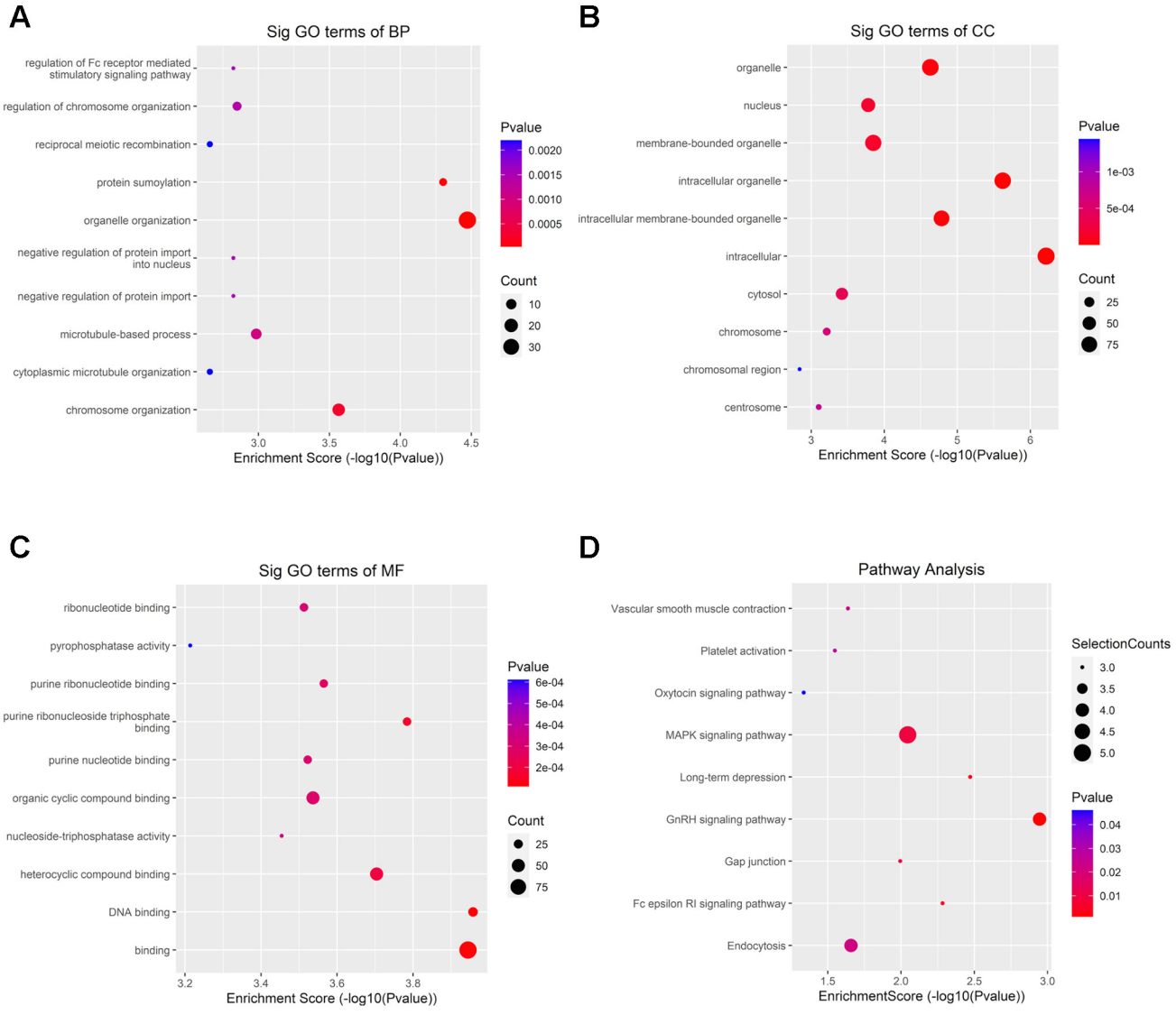

Figure 4. GO and KEGG analysis of significantly differential expression. The top 10 enriched A) biological processes, B) cellular components, and C) molecular functions were discovered by GO analysis. D) The most significantly enriched pathway was unveiled by KEGG analysis.

is estimated between 20 and 22% [39]. The noninvasive detection of adenoma is still under study. A multitarget stool DNA test was recommended as a new CRC screening method in the updated guideline for adults elder than 45 years with an average risk of CRC [40]. As a potential minimally invasive way, a liquid biopsy will play a greater role in clinical application. High-throughput sequencing has been applied to liquid biopsy. Exosomes could be released by cancer cells and act as intercellular mediators of oncogenic information [41]. Compelling evidence has indicated that cancer-derived exosomal miRNAs play an oncogenic role in CRC growth, chemoresistance, and metastasis, and could be potential diagnostic biomarkers for CRC [42–45]. Moreover, the levels of exosomal miRNAs were higher in several tumor cell lines compared to non-malignant cell lines in accordance with their findings in serum exosomes of CRC patients and healthy individuals [46, 47]. Many studies have focused on ncRNAs in exosomes, and observed an upregulation of some ncRNAs in exosomes of cancer subjects when compared to healthy control [48, 49].

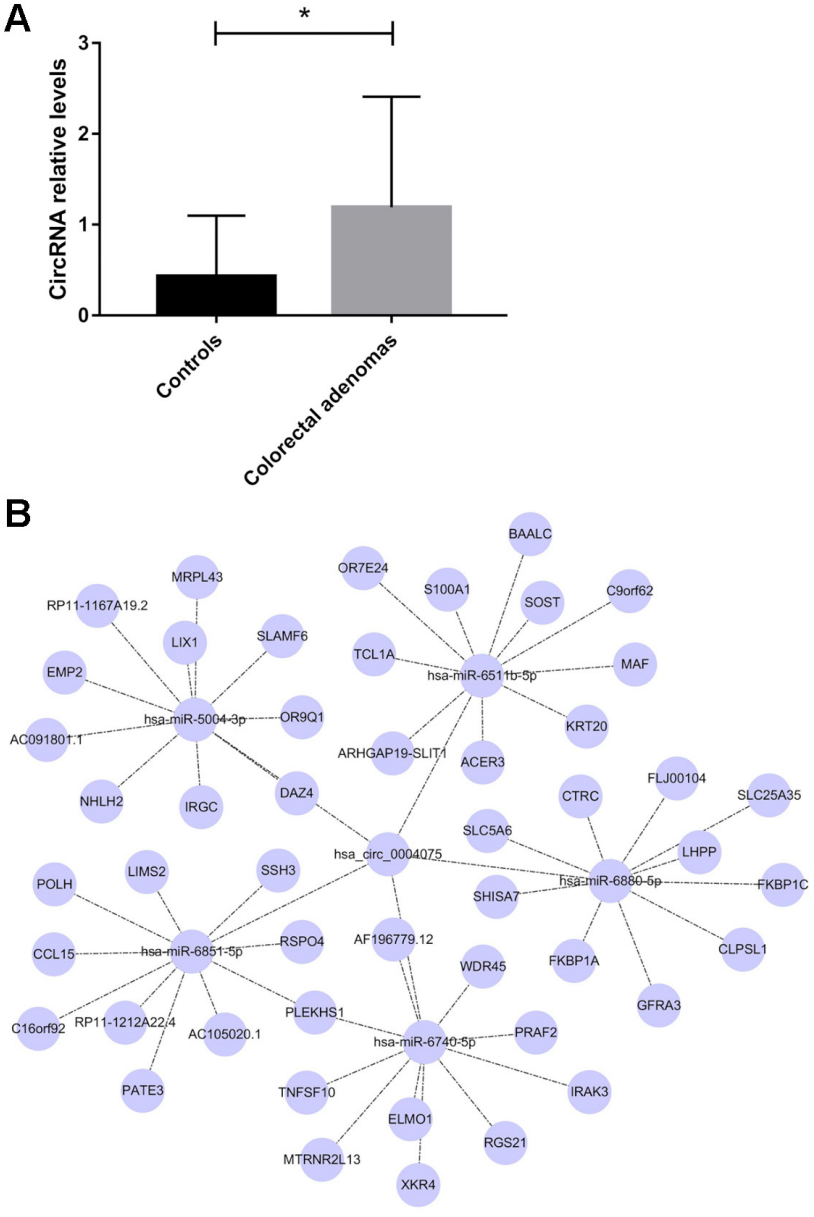

Figure 5. Prediction and analyses of circRNA-miRNA-mRNA network. A) Validation of the RNA sequencing results by qRT-PCR. B) The hsa_circ_0004075-mediated ceRNA network.

However, the role of exosomes in colorectal adenomas still needs to be further clarified.

Exosomes can be transferred from tumor cells to the peripheral circulation and act as carriers of information in intercellular communication [50, 51]. The differentially expressed exosomal circRNAs in the plasma may play a pivotal role in affecting tumor growth and proliferation and mediating the crosstalk between the tumor and tumor-associated cells. To explore the mechanism of plasma exosomes in colorectal adenomas and healthy control, the exosomes were isolated from the plasma of 3 colorectal adenomas patients and 3 healthy individuals. We then identified these exosomes using TEM, WB, and NTA (Figures 1A–1C).

Alterations in components of exosomes in plasma are related to the pathological processes of various diseases, including cancer [52]. CircRNAs form a class of post-transcriptional regulators, which compete with other RNAs for binding by miRNAs and RBPs and may generally function in modulating the concentration of RBPs, RNAs, or their binding sites [53]. It was reported that the expression patterns of circRNAs varied between tumor and normal cells [54, 55]. It is necessary to identify anti-degradation biomarkers for clinical screening analysis. Vo et al. have reported that circRNAs were more stable than linear RNA in plasma after incubation, and circRNAs could be reliably detected in urine samples [56]. These unique characteristics could present them as a promising biomarker for early detection and prognosis. Accumulating evidence showed that some circRNAs are involved in the malignant progression of colorectal cancer, including malignant proliferation, chemotherapy resistance, and metastasis [57]. Furthermore, the potential functions of circRNAs have been investigated extensively, including sponging miRNAs, encoding peptides, binding to RBPs, promoting gene transcription, and regulating alternative splicing [58, 59]. Additionally, miRNAs, lncRNAs, and circRNAs are harbored by released exosomes and play a crucial role in carcinogenesis as a messenger by carrying and delivering oncogenic molecules in cellular communication [60, 61]. In recent years, more and more attention has been paid to the circRNAs of exosomes as diagnostic and therapeutic targets, and the expression profiles of some disease-related exosomes have been identified. Uratani et al. demonstrated the potential role of total serum miR-21, miR-29a, and miR-92a in the early detection of colorectal adenomas and cancers [62]. CircRNAs and exosomal circRNAs have the potential diagnostic values for gastrointestinal (GI) malignancies and lung cancer [63, 64]. Nevertheless, the role of exosomal circRNAs in colorectal adenomas has not been established. In the present study, we have analyzed the clinical correlation of plasma exosomal circRNAs in colorectal adenomas compared to the healthy control group based on the results of the RNA sequence. Our results uncovered that 413 circRNAs were differentially expressed between colorectal adenomas patients and healthy control (Figure 2D).

New studies revealed that exosomal circRNAs are involved in the malignant transformation, and achieve the efficient transmission of phenotypical changes, thereby promoting malignant progression [65–67].

We carried out this research to identify an intracellular communication mechanism that hasn't been explained before in colorectal adenomas.

We focused on the characteristics of exosomal circRNAs that are significantly upregulated in colorectal adenomas, as they are likely to be associated with the progression. To investigate the role of the differentially expressed circRNAs in colorectal adenomas, we performed GO and KEGG pathway analyses to predict their potential functions. GO analysis uncovered that these circRNAs were significantly associated with the organelle organization, intracellular organelle, and DNA binding (Figures 3A–3D). Therefore, we suspected that these circRNAs might be related to the regulation of the signaling pathway. Similarly, the highly relevant signaling pathways are mainly involved in the GnRH signaling pathway and MARK signaling pathway from the KEGG pathway analyses.

The previous study has indicated that there are six means of circRNAs functions, including serving as miRNA sponges, protein sponges, protein function enhancers, protein scaffolds, protein recruiters, and translation templates [68]. The competitive endogenous RNA (ceRNA) hypothesis supposed that RNAs may affect miRNAs activity by competing miRNAs binding sites, thereby regulating miRNAs target gene expression [69]. There are various miRNA binding sites in circRNAs, which would attenuate miRNAs activity and upregulate the level of miRNAs target genes. It provides new insights into exploring the mechanisms of the circRNAs. In our study, the potential miRNA targets of hsa_circ_0004075 were predicted by the database. The results showed negative correlations between hsa_circ_0004075 and related miRNAs. These results revealed that hsa_circ_0004075 may play a central role in the occurrence and development of colorectal cancer. Thus, further investigations are required to verify the utility of exosomal circRNAsHowever, it must be acknowledged that the sample size is not yet enough, which is the limitation of this study. In order to confirm the diagnostic efficiency of plasma exosomal hsa_circ_0004075, a large-scale, multi-center sample study is necessary. Due to our short study time and limitations on patient information, we did not find suitable data to explore the correlations between plasma exosomal hsa_circ_0004075 and adenoma recurrence. Therefore, it is necessary to establish a long-term follow-up system to demonstrate whether the level of plasma exosomal hsa_circ_0004075 affects adenoma recurrence. We are confident that the current results indicated that plasma exosomal hsa_circ_0004075 may be a potential biomarker for colorectal adenomas. In addition, qRT-PCR was used to detect the expression of exosomal hsa_circ_0004075 in plasma of fresh samples, which is convenient for clinical application.

Collectively, based on our results, an initial landscape of the exosomal circRNAs differential expression in plasma from colorectal adenomas was presented through high-throughput sequencing and was validated using qRT-PCR. The results indicated that hsa_circ_0004075 has the potential as a novel biomarker and therapeutic target for colorectal adenomas. Our study also broadens the understanding of exosomal circRNAs in the pathogenesis of colorectal adenomas. Further research is needed to explore the underlying mechanisms of hsa_circ_0004075 in colorectal cancer evaluation in the future.

Acknowledgments: Our work is supported by the National Natural Science Foundation of China (81900461), and the Zhejiang Provincial Natural Science Foundation of China (LQ19H160022).

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