Original Paper

Circ-ANAPC7 is Upregulated in Acute Myeloid Leukemia and Appears to Target the MiR-181 Family

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Key Words

Acute Myeloid Leukemia • Circular RNA • Circ-ANAPC7 • Microarray

Abstract

Background/Aims: Circular RNAs (circRNAs) are a family of novel non-coding RNAs associated with various diseases, especially cancer. Recent studies have demonstrated that circRNAs participate in pathogenesis mainly by acting as microRNA (miRNA) sponges. The expression profile of circRNAs in acute myeloid leukemia (AML) has rarely been reported. **Methods:** Profiles of circRNAs were analyzed using an Arraystar human circRNA microarray with 5 bone marrow samples from patients with newly diagnosed AML and 5 from patients with iron-deficiency anemia. Quantitative reverse transcription PCR was used to validate the expression pattern of circRNAs. Furthermore, circRNA-miRNA network, Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were applied. **Results:** CircRNA microarray analysis revealed that 698 circRNAs were differentially expressed in AML patients, with 282 circRNAs found to be upregulated and 416 to be downregulated. Quantitative reverse transcription PCR showed that circ-ANAPC7 was significantly upregulated in AML. Bioinformatics analysis predicted that circ-ANAPC7 acts as a sponge for the miR-181 family, KEGG analysis revealed that it is associated with cancer-related pathways, and GO analysis indicated that most of its target genes are involved in biological processes. **Conclusions:** These findings show that circ-ANAPC7 is a promising biomarker for AML, and that it might participate in AML pathogenesis by acting as a sponge for the miR-181 family.

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Introduction

Acute myeloid leukemia (AML) is a group of hematopoietic malignancies characterized by a poor prognosis. The pathogenesis of AML is closely associated with various genetic abnormalities that interfere with the growth, proliferation, and differentiation of normal hematopoietic progenitor cells [1, 2]. These abnormalities often lead to rapidly occurring fatal infections, bleeding, or organ infiltration [3]. Overwhelming evidence has established a regulatory role for protein-coding genes in the tumorigenesis, maintenance, and progression of AML, while non-coding RNAs (ncRNAs), such as long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), have also been shown to be of great importance in AML [4].

CircRNAs are a family of novel ncRNAs that used to be identified as a by-product of abnormal RNA splicing with no biological function. More recently, accumulating evidence has shown that they play a crucial role in post-transcriptional regulation *in vivo* [5, 6]. CircRNAs are characterized by a covalently closed loop structure lacking 3' and 5' ends. They are highly stable and mainly reside in the cytoplasm, and thus have the potential to be used as biomarkers [7]. A number of studies have found that circRNAs, such as CDas1R, have an important function in the pathogenesis of several diseases [5, 8, 9]. However, little is known about the role of circRNAs in human cancer.

CircRNAs have also been observed to act as microRNA (miRNA) sponges, which contribute to the regulation of gene expression. miRNAs are ~23-nt ncRNAs that are implicated in numerous biological processes [10]. For instance, several studies have demonstrated that they function as guide RNAs leading to RNA silencing, and miRNA dysfunction is associated with the development of diseases such as leukemogenesis [11]. miRNAs also participate in post-transcriptional regulation in AML, leading to a cascade of downstream effects and indicating the potential for targeted therapeutic strategies. CircRNA-based therapy is a novel approach to modulate dysregulated miRNAs in AML. Hence, in this study, we investigated the circRNA-miRNA regulatory network in AML.

We first explored the expression profile of circRNAs in 5 AML samples and 5 iron-deficiency anemia (IDA) samples (control group) that were collected from the bone marrow of patients. Then, we validated one circRNA (hsa_circRNA_101141) derived from the ANAPC7 gene locus, termed circANAPC7, which was frequently upregulated in patients with AML. Finally, we found that circANAPC7 may act as a miR-181 sponge by bioinformatics analysis.

Materials and Methods

Patients and specimen collection

This study involved 87 patients diagnosed with AML and 45 patients diagnosed with IDA as a control group. All individuals were of Han Chinese ethnicity and were recruited from the Second Affiliated Hospital of Xi'an Jiaotong University. None of the patients had previously undergone chemotherapy. All specimens were obtained from bone marrow aspiration, and after removal were immediately preserved at -80°C until required. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University (No. 2015181) and met international standards for patient confidentiality. Written informed consent was obtained from all patients.

Total RNA extraction

Total tissue RNA was extracted from the AML and IDA samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and concentration of RNA samples were determined with a NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

Microarray analysis

We selected five AML specimens and five IDA specimens for microarray analysis. Total RNA was treated with RNase R to enrich circular RNAs. The enriched circular RNAs were then amplified and transcribed into fluorescent cRNA utilizing random primers according to the Arraystar Super RNA Labeling protocol

(Arraystar, Inc., Rockville, MD, USA). The labeled circRNAs were hybridized onto an Arraystar Human circRNA Array (8×15K, Arraystar, Inc.), and then incubated at 65°C for 17 h. After washing the array with gene expression wash buffer (Agilent Technologies, Santa Clara, CA, USA), the slide was scanned with an Agilent Scanner G2505C.

CircRNA microarray data analysis

Data were extracted using Agilent Feature Extraction software. Data processing, including quantile normalization, was performed using R software. CircRNAs that were significantly differentially expressed between the AML and IDA groups were identified using a fold-change cut-off value or through volcano plot filtering. The prediction of miRNA targets for circRNAs and circRNA-miRNA interactions was conducted using the TargetScan and miRanda algorithms.

Validation of candidate circRNAs using quantitative reverse transcription polymerase chain reaction

Candidate circRNAs were selected for validation if they met the following criteria: 1) the raw intensity of all tested samples was greater than 500; 2) the P-value of the circRNAs between AML and IDA was <0.01; 3) the fold change in expression between AML and IDA was >2.5; and 4) the miRNAs in the predicted circRNA-miRNA-mRNA/lncRNA network has been proven to be associated with AML in previous studies. cDNA was synthesized by reverse transcription using a PrimeScript RT Master Mix with random primers according to manufacturer's protocols (TaKaRa Bio, Shiga, Japan). Then, quantitative reverse transcription PCR (qRT-PCR) was performed using SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa) following the manufacturer's instructions. Divergent primers were synthesized by Invitrogen (Shanghai, China) and the sequences are shown in Table 1.

CircRNA-microRNA co-expression network

Validated candidate circRNAs were used as seeds to enrich a circRNA-miRNA-mRNA/lncRNA network according to the analysis of starBase v2.0 (<http://starbase.sysu.edu.cn/>) [12]. Cytoscape (<http://www.cytoscape.org/>) was applied to build a circRNA-miRNA-mRNA/lncRNA network. miRTarBase (<http://mirtarbase.mbc.ntu.edu.tw/>) was used to extract experimentally validated targets of the top 5 predicted miRNAs for validated candidate circRNAs [13].

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were carried out using the DAVID analysis tool (<https://david.ncifcrf.gov/>) to define further the biological pathways of the predicted miRNAs. Gene Ontology (GO) analysis was also performed to clarify the functional roles of miRNA target genes.

Statistical analysis

Results are reported as means ± standard deviation for triplicate measurements. Significant differences between groups were estimated by the Mann-Whitney test using SPSS v.23.0 software (IBM, Chicago, IL, USA). P < 0.05 was considered statistically significant.

Results

Profiling of differentially expressed circRNAs in AML patients

The expression of 7441 human circRNAs was quantitated for the AML and IDA samples using a microarray platform. Hierarchical clustering and scatter plot visualization showed that circRNA expression levels were variable and distinguishable among the samples (Fig. 1).

Table 1. qRT-PCR primer sequences

Primer name	Primer FW (5' – 3')	Primer RV (5' – 3')
β-actin(H)	GTGGCCGAGGACTTTGATTG	CCTGTAACAACGCATCTCATATT
hsa_circRNA_101141	GGGAGCAGCACTTAGGAACAT	AAAGCTGGTACTTCTGAGGTGG
hsa_circRNA_103164	AGGACGAGCAGGGTGCAGAA	CTGACAGCGCAGTTTGAGGG

A total of 698 circRNAs were differentially expressed (fold change ≥ 2.0 , $P < 0.05$) between the AML and IDA samples, with 282 circRNAs found to be upregulated and 416 to be downregulated by more than 2-fold in AML patients compared with IDA patients. Analysis of the chromosomal distribution of circRNAs revealed that chromosome 1 had the greatest number of differentially expressed circRNAs, while mitochondrial DNA and chromosome 13 had the lowest number of differentially expressed circRNAs (Fig. 2a). The classification of all differentially expressed circRNAs is shown in Fig. 2b. Most differentially expressed circRNAs are transcribed from protein-coding exons, some are derived from introns, while a small number come from other sources. These data suggest that the expression of circRNAs in the bone marrow of AML patients differs from that in IDA patients.

Fig. 1. CircRNA microarray expression data. (A) Hierarchical clustering indicates differences in circRNA expression profiling between the AML and control groups. (B) A scatter plot was used to assess circRNA expression variation between the AML and control groups. The values of the X and Y axes in the scatter plot are the normalized signal values of the samples (log2 scaled). The middle green line indicates no difference between both groups. CircRNAs above the top green line or below the bottom green line indicate more than a 2-fold changes. (C) A volcano plot was applied to visualize expression profiling between both groups. The vertical green lines refer to a 2.0-fold (log2 scaled) increase and decrease, respectively. The horizontal green line corresponds to a P-value of 0.05 (-log10 scaled). Each red point represents a differentially expressed circRNA with statistical significance.

Fig. 2. Distribution and category of differentially expressed circRNAs. (A) Distribution of differentially expressed circRNAs in human chromosomes. (B) CircRNA category is shown in the bar diagram. Most differentially expressed circRNAs originate from exons.

Fig. 3. Validation of circ-ANAPC7 expression by quantitative reverse transcription PCR. Circ-ANAPC7 is significantly upregulated in AML patients ($P < 0.001$).

Fig. 4. CircRNA-miRNA-mRNA/lncRNA co-expression network. The red, yellow, purple, and blue nodes represent circRNA, miRNA, mRNA, and lncRNA, respectively.

Fig. 5. Prediction of circRNA and miRNA interactions. The interactions of circ-ANAPC7 and the top 5 target miRNAs were predicted based on TargetScan and miRanda. The prediction for the interaction of circ-ANAPC7 with the miR-181 family was completely consistent in both databases.

Table 2. Validated targets for circ-ANAPC7-related miRNAs

miRNA	Reporter assay	Western blot	qRT-PCR	Total
hsa-miR-181a-5p	RGSS RALA PRKCD ATM TERT DDIT4 HIPK2 SIRT1	RGSS RALA PRKCD ATM CD4 BCL2 WIF1 RAP1B	RGSS RALA PRKCD ATM CD4 TERT DDIT4 HIPK2	RGSS RALA PRKCD ATM TERT DDIT4 HIPK2 SIRT1 ATG5 BCL2 WIF1
	ATG5 BCL2 WIF1 RAP1B E2F5 NLK GATA6 CDX2	RGSS RALA PRKCD ATM CD4 BCL2 WIF1 RAP1B	SIRT1 ATG5 BCL2 WIF1 RAP1B E2F5 RNF2 MAP2K1	RAP1B E2F5 NLK GATA6 CDX2 BCL2L11 PRAP1 FOS MTMR3 MCL1
	BCL2L11 PRAP1 FOS MTMR3 MCL1 XIAP GPR78	E2F5 RNF2 MAP2K1 ABCG2 MAPK1 ZNF763	ABCG2 MAPK1 PROX1 BCL2L11 KLF6 DDX3X KRAS	XIAP GPR78 PPP3CA TWIST1 RNF2 MAP2K1 ABCG2 MAPK1 ZNF763
	PPP3CA TWIST1 RNF2 MAP2K1 ABCG2 MAPK1	PROX1 BCL2L11 KLF6 DDX3X KRAS PBX3	PBX3 TIMP1 PGR COL16A1 TGFBRAP1 TGFBR1 HRAS	PROX1 KLF6 DDX3X KRAS PBX3 TIMP1 PGR COL16A1 TGFBRAP1
	ZNF763 PROX1 KLF6 DDX3X KRAS PBX3 TIMP1	TIMP1 PGR COL16A1 TGFBRAP1 TGFBR1 HRAS	CDKN1B C12ORF29 PHACTR2 SASH1 PHLDA1	TGFBR1 HRAS DUSP6 PTPN11 DUSP5 PTPN22 NOTCH1 CDKN1B
	PGR COL16A1 TGFBRAP1 TGFBR1 HRAS DUSP6	DUSP6 PTPN11 DUSP5 PTPN22 NOTCH1	MOSPD1 SRGAP1 UBL3 PHACTR4 EREG IFNG AHR	IFNG KAT2B PLAG1 CD4 AHR STAT3 C12ORF29 PHACTR2 SASH1
	PTPN11 DUSP5 PTPN22 NOTCH1 CDKN1B IFNG	CDKN1B IFNG AHR STAT3 KAT2B PLAG1	STAT3	PHLDA1 MOSPD1 SRGAP1 UBL3 PHACTR4 EREG
	KAT2B PLAG1			
	NLK GATA6 CDX2 TIMP3 PLAG1 GRIA2 VSNL1	TIMP3 PLAG1 BCL2 KAT2B TCL1A MAP3K10	TIMP3 BCL2 TCL1A MAP3K10 CYLD RNF2 TMED7	NLK GATA6 CDX2 TIMP3 PLAG1 GRIA2 VSNL1 BCL2 KAT2B TCL1A
	BCL2 KAT2B TCL1A CYLD RNF2 TMED7 SIRT1	RNF2 TMED7 E2F1 LATS2 PBX3 ATM CREB1	LATS2 PBX3 CREB1 RAP1B ADCY9 FOS SIX2	CYLD RNF2 TMED7 SIRT1 CBX7 XIAP MCL1 IGFBP1 E2F1 LATS2 PBX3
hsa-miR-181b-5p	CBX7 XIAP MCL1 IGFBP1 E2F1 LATS2 PBX3 ATM	RAP1B ADCY9 FOS NFIA SIX2		ATM CREB1 RAP1B ADCY9 MAP2K1 FOS SIX2 MAP3K10 NFIA
	CREB1 RAP1B ADCY9 MAP2K1 FOS SIX2			
hsa-miR-181d-5p	BCL2 HRAS MGMT RAP1B	BCL2 HRAS MGMT RAP1B	BCL2 HRAS MGMT RAP1B	BCL2 HRAS MGMT RAP1B
hsa-miR-338-3p	PLA2G4B SMO CCND1 PREX2 SSX2IP NRP1	UBE2Q1 NOVA1 MAP1A DAB2IP ZNF238 SMO	UBE2Q1 NOVA1 MAP1A DAB2IP ZNF238 PLA2G4B	PLA2G4B SMO CCND1 PREX2 SSX2IP NRP1 MACC1 ZEB2 UBE2Q1
	MACC1 ZEB2	MMP2 MMP9 PREX2 SSX2IP NRP1 MACC1 ZEB2	NOVA1 MAP1A DAB2IP ZNF238 MMP2 MMP9	
hsa-miR-526b-5p	none	none	none	none

Validation of differentially expressed circRNAs by qRT-PCR

Considering the lower expression level of circRNAs and difficulty in over-expression functional test techniques, we selected only upregulated circRNAs for further validation according the criteria described in the Materials and Methods. Finally, circ-ANAPC7 (hsa_circRNA_101141) and circ-PI4KA (hsa_circRNA_103164) were tested by qRT-PCR in 87 AML and 45 IDA bone marrow samples. As shown in Fig. 3, circ-ANAPC7 was significantly upregulated in AML patients, while circ-PI4KA showed a trend toward overexpression in AML patients, but this was not significant (data not shown). There was no significant difference in age and sex between the AML and IDA groups (data not shown).

miRNA prediction and bioinformatics analyses

Bioinformatics analysis showed that circ-ANAPC7 harbored miRNA response elements (MREs) for hsa-miR-181a-5p, hsa-miR-181b-5p, hsa-miR-181d-5p, hsa-miR-338-3p, and hsa-miR-526b-5p (Fig. 4). This was confirmed by the starBase database for hsa-miR-181a-5p, hsa-miR-181b-5p, and hsa-miR-181d-5p MREs, suggesting that circ-ANAPC7 might regulate miRNA-associated target genes in AML by acting as a sponge for the miR-181 family. To clarify the biological function of these five miRNAs, we extracted their validated targets from miRTarBase. miR-181a was shown to

Table 3. KEGG pathways

KEGG pathway	p-value	#genes	#miRNAs	Gene
Pathways in cancer	0.000995	11	4	WNT16 BCL2 CDKN1B KRAS MMP2 PTGS2 WNT3A MAP2K1 SMO WNT2 CCDC6
MAPK signaling pathway	0.032874	8	3	PLA2G4B TAB2 KRAS BDNF GADD45G NLK MAP2K1 PLA2G4C
HTLV-I infection	0.034562	8	2	WNT16 KRAS NFYB ATM NRP1 KAT2B WNT3A WNT2
Long-term depression	5.63E-08	6	3	PLA2G4B GNAI3 KRAS GRIA2 MAP2K1 PLA2G4C
Melanogenesis	0.001262	6	1	WNT16 GNAI3 KRAS WNT3A MAP2K1 WNT2
Serotonergic synapse	0.00542	6	2	PLA2G4B GNAI3 KRAS PTGS2 MAP2K1 PLA2G4C
VEGF signaling pathway	0.000628	5	2	PLA2G4B KRAS PTGS2 MAP2K1 PLA2G4C
GnRH signaling pathway	0.003231	5	2	PLA2G4B KRAS MMP2 MAP2K1 PLA2G4C
Hedgehog signaling pathway	0.001553	4	2	WNT16 WNT3A SMO WNT2
Glycerophospholipid metabolism	0.003005	4	2	PLA2G4B LCLAT1 LPGAT1 PLA2G4C
Basal cell carcinoma	0.003005	4	2	WNT16 WNT3A SMO WNT2
Fc epsilon RI signaling pathway	0.009002	4	2	PLA2G4B KRAS MAP2K1 PLA2G4C
Prostate cancer	0.009311	4	3	BCL2 CDKN1B KRAS MAP2K1
Thyroid cancer	0.000609	3	1	KRAS MAP2K1 CCDC6
Dorso-ventral axis formation	0.000995	3	1	NOTCH2 KRAS MAP2K1
Cocaine addiction	0.009002	3	2	GNAI3 BDNF GRIA2
Bladder cancer	0.010895	3	2	KRAS MMP2 MAP2K1
Notch signaling pathway	0.014492	3	2	NOTCH2 LFNG KAT2B

Fig. 6. KEGG pathway analysis.

Fig. 7. Circ-ANAPC7 is related to pathways in cancer.

have the most targets, while miR-526 had none. The details of the validated targets of the five miRNAs are shown in Table 2.

To investigate further the molecular mechanism of circRNAs, the circ-ANAPC7-miRNA-mRNA/lncRNA network was constructed by Cytoscape (Fig. 5). Moreover, because these miRNAs have been suggested to play important roles in oncogenesis, KEGG pathway enrichment analysis was conducted using DAVID (Fig. 6). The five miRNAs appeared to be strongly associated with cancer-related pathways (Fig. 7). Table 3 lists the combinatorial effect of the five miRNAs in the top 20 pathways and their target genes. GO enrichment analysis of circ-ANAPC7 target genes revealed that their properties could be defined as molecular function and cellular component (data not shown). These processes are closely associated with AML tumorigenesis.

Discussion

AML is a heterogeneous clonal malignancy characterized by the failure of hematopoietic proliferation [14]. Within China, the incidence of leukemia is approximately 4.44 per 100,000 men and 3.09 per 100,000 women [15]. Despite rapid advances in AML therapy, including the development of novel drug targets and transplantation, treatment has remained stagnant for the past few decades, with most patients relapsing and dying of complications [16]. Even with current treatment, the majority of patients older than 65 years will die of AML within 1 year [17, 18]. To address this issue, research has focused on the identification of novel targets for AML therapy.

A wide range of evidence has indicated that ncRNAs such as circRNAs play an important role in leukemogenesis [19-21]; thus, circRNA-based therapy is a feasible approach for the treatment of AML. Aberrant circRNA expression has been associated with the malignant

behavior of cancers [22, 23], but few studies have reported the potential roles of circRNAs in AML.

Here, we explored differences in the expression patterns of circRNAs between AML and IDA patients. Microarray analysis identified 282 circRNAs that were upregulated and 416 that were downregulated in AML, suggesting their potential involvement in disease pathogenesis. We further demonstrated by qRT-PCR that circ-ANAPC7 was significantly upregulated in AML, indicating that it could act as an oncogenic circRNA. Bioinformatics analysis predicted that circ-ANAPC7 functioned as a sponge for the miR-181 family because it harbored several MREs for this family. These results are consistent with our previous hypothesis that circRNAs are oncogenic in AML, and suggest that circ-ANAPC7 could be used as a biomarker for AML diagnosis or as a novel drug target.

The miR-181 family comprises miR-181a, miR-181b, miR-181c, and miR-181d, which are encoded by three different transcripts located on three chromosomes. All miR-181 family members contain similar seed sequences that differ by fewer than four nucleotides [24]. Previous studies have demonstrated that the miR-181 family regulates the differentiation of B cells, T cells, and natural killer cells during normal hematopoiesis [25-27]. miR-181a was reported to regulate non-small cell lung cancer proliferation and migration through targeting VCAM-1 [28]. In some tumors, the miR-181 family was shown to target the anti-apoptotic genes BCL2 and MCL1, leading to apoptosis and the inhibition of tumor growth. Dysregulation of the miR-181 family was also shown to be closely associated with the pathogenesis and prognosis of AML. Additionally, members of the miR-181 family were demonstrated to mediate several cancer-related pathways, such as the RAS/MAPK pathway, thus acting as tumor suppressors in myeloid leukemogenesis [29, 30]. Therefore, targeting the circ-ANAPC7-miR-181 axis may be a novel therapeutic approach for AML.

This study has a number of limitations. First, we chose IDA patients as our control group because it is difficult to obtain bone marrow samples from healthy individuals. Second, we validated circ-ANAPC7 as a competitive endogenous RNA of the miR-181 family by only bioinformatics analysis, and not by experimental means. Finally, we did not elucidate the clinical significance of circ-ANAPC7 because of the short follow-up period. Further studies are therefore needed to determine the clinical significance and underlying mechanisms of circ-ANAPC7 in the pathogenesis of AML.

Conclusion

This study shows that circ-ANAPC7 is a promising biomarker for AML, and that it might participate in the pathogenesis of AML by acting as a sponge for the miR-181 family.

Abbreviations

miR (or miRNA; microRNA); circRNA; circular (RNA); ncRNA; non-coding (RNA); AML (acute myeloid leukemia); IDA; iron-deficiency (anemia); lncRNA; long (non-coding RNA); qRT-PCR; quantitative (reverse transcription PCR); GO; Gene (Ontology); KEGG; Kyoto (Encyclopedia of Genes and Genomes); MRE; miRNA (response element).

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All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Disclosure Statement

No competing financial interests exist.

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