Circular RNA expression profiles in the plasma of patients with infantile hemangioma determined using microarray analysis

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Abstract. Circular RNAs (circRNAs) are noncoding RNAs that have important roles in tumor progression. Previous studies have examined the circRNAs involved in infantile hemangioma (IH) tumors. The present study compared the circRNA levels in plasma samples from patients with IH and control individuals. The circRNA expression profiles were determined using microarray in three pairs of plasma samples from patients with proliferative IH and healthy control subjects. Expression of the identified differentially expressed circRNAs was verified using reverse transcription-quantitative PCR (RT-qPCR) and a bioinformatics analysis was performed to predict the microRNAs targeted by the validated circRNAs. From the circRNA expression profiles in the plasma of patients with IHs, 128 differentially expressed circRNAs were identified, of which 72 were upregulated and 56 were downregulated. The downregulated expression of three circRNAs [Homo sapiens (hsa)_circRNA_101566, hsa_circRNA_103546 and hsa_circRNA_103573] was verified using RT-qPCR. Gene Ontology term and Kyoto Encyclopedia of Genes and Genomes pathway analyses indicated that all identified networks participated in angiogenesis and tumor formation and progression. It was determined that hsa_circRNA_101566, which is able to regulate the mTOR signaling pathway, may be an important regulatory molecule in IH development and that targeting of hsa_miR_520c is able to indirectly regulate the vascular endothelial growth factor signaling pathway. Further studies are required to clarify these effects and the underlying mechanisms.

Introduction

Infantile hemangioma (IH) is the most common type of soft tissue tumor in infants, with an incidence of 4-10% (1,2). The growth of IH typically increases rapidly during the first three months, particularly between the first five and eight weeks of life, and ~80% of its maximum growth is completed by the age of three months (3,4). Although most IHs resolve naturally without treatment, 10-15% of cases have complications, such as obstruction, ulceration or disfigurement, and require intervention. IH results from angiogenesis and angiogenesis-related disorders. However, the factors that trigger IH development have remained to be elucidated. Therefore, studies investigating the molecular mechanisms of the tumorigenesis and progression of IH are required.

Circular RNA (circRNA) is a type of noncoding RNA (ncRNA) composed of transcripts from exons. It was first discovered by Sanger et al (5) in a virus by using electron microscopy in 1976. Over the last few years, circRNAs have been indicated to have multiple functions, including acting as microRNA (miRNA/miR) sponges, influencing gene expression by regulating splicing or transcription and interacting with RNA-binding proteins (6-8). An increasing number of studies have demonstrated the functions of circRNAs in various diseases. Furthermore, circRNAs may serve as prognostic and diagnostic biomarkers for various diseases (9). A previous study by our group, in which the profiles of circRNAs in IH and adjacent normal tissues were compared, revealed that circRNAs participate in angiogenesis and vascular development-related biological processes (10). However, to the best of our knowledge, plasma circRNAs have so far remained to be systematically evaluated in IH. As circRNAs are excreted from cells in exosomes, their recovery from the blood is a viable option.

The present study aimed to determine the circRNA profiles of three pairs of plasma samples from patients with proliferative IH and healthy control individuals using high-throughput microarray and explore the roles of these circRNAs in IH using bioinformatic methods. The results enhance the current understanding of the roles of circRNAs in the pathogenesis of
IH and may aid in the discovery of blood-based biomarkers for diagnosing IH.

Materials and methods

Patient samples. In the present study, the inclusion criteria were as follows: Proliferative IH diagnosed by two independent doctors according to clinical and pathological features (red soft lump that appears within 3 months of birth and rapidly increases in size, with an elevated skin temperature) (3) and Doppler ultrasound (frequently subcutaneous, with variable echogenicity; intralosomal vessels not visible on gray-scale imaging; high vascular density on color Doppler; increased echogenicity and decreased vascularity with involution) (11). Furthermore, only patients aged 3-6 months were considered to ensure that the IHs were still in the proliferative phase. The exclusion criteria were as follows: i) Administration of any treatment for IH prior to participating in the study and ii) the presence of another disease. Blood samples from a total of 17 patients with IH were collected at the Department of Plastic Surgery of Shandong Provincial Hospital (Jinan, China) between May 2018 and April 2019. In addition, blood samples were collected from 17 normal age‑matched healthy volunteers. The volunteers came from the children in the physical examination center of Shandong Provincial Hospital (Jinan, China) and blood was collected with the consent of their parents/guardians. Plasma was obtained from the blood samples via centrifugation and stored at -80°C. A total of three randomly selected sample pairs were used for further validation of the microarray results. The other 14 sample pairs were used for microarray analysis. The other 14 sample pairs were used for validation of the microarray data using reverse transcription-quantitative PCR (RT-qPCR). The total RNA from the three paired plasma samples of IH cases and healthy children used for microarray analysis was isolated using the TRIzol reagent and reverse-transcribed (2X PCR master mix; Arraystar, Inc.; 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec) to synthesize cDNA using a GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

RNA isolation and quality control. RNA from each sample was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The purity and concentration of the total RNA samples were determined using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). RNA integrity was assessed through electrophoresis on a denaturing agarose gel.

Microarray. Sample labeling and array hybridization were performed according to the manufacturer's protocol (Arraystar human circular RNA v2; design ID 074301). In brief, total RNAs were digested with RNase R (Epitcentre; Illumina, Inc.) to remove linear RNAs and enrich circRNAs. The enriched circRNAs were amplified and transcribed into fluorescent complementary (c)RNAs using a random priming method according to the Arraystar's protocol (Super RNA Labeling Kit; Arraystar, Inc.). The labeled cRNAs were purified using an RNeasy Mini Kit (Qiagen GmbH). The concentration and specificity of the labeled cRNAs (pmol Cy3/µg cRNA) were measured using a NanoDrop ND-1000. Thereafter, 1 µg of each labeled cRNA was fragmented by adding 5 µl of 10X blocking agent and 1 µl of 25X fragmentation buffer, followed by heating at 60°C for 30 min. Subsequently, 25 µl of 2X hybridization buffer was added to dilute the labeled cRNA. Next, 50 µl of hybridization solution was dispensed into the gasket slide and assembled on the circRNA expression microarray slide. The slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven (Agilent Technologies, Inc.). The hybridized arrays were washed, fixed and scanned using an Agilent Scanner (G2505C; Agilent Technologies, Inc.).

Validation of microarray data using reverse transcription-quantitative PCR (RT-qPCR). The total RNA from the three paired plasma samples of IH cases and healthy children used for microarray analysis was isolated using the TRIzol reagent and reverse-transcribed (2X PCR master mix; Arraystar, Inc.; 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec) to synthesize cDNA using a GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the kit's protocols. GAPDH was selected as the internal reference. All cdNAs were assembled in the Viia 7 real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) (2X Master Mix, 5 µl; 10 µM PCR specific primer F, 0.5 µl; 10 µM PCR specific primer R, 0.5 µl; water was added to create the total volume of 8 µl). The reaction was performed as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec. All targets and references were evaluated in triplicate wells. The relative expression levels of each target circRNA were calculated using the 2^ΔΔCt method (12). Primer sequences are presented in Table II. The identified circRNAs, which were consistent with the regulatory direction of the microarray results and significantly differentially expressed, were further verified in the remaining 14 sample pairs.

Construction of circRNA-miRNA-mRNA networks. The circRNA-targeted miRNAs were predicted using Arraystar's homemade miRNA target prediction software (Arraystar, Inc.) based on the data obtained from TargetScan, miRanda and statistical analysis (methods listed in Data S1). The network was constructed using Cytoscape software (version 2.8.3) (https://cytoscape.org/). Gene ontology (GO) term analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (http://david.abcc. nciifcr.gov/) to explore the potential functions of the target genes in the categories biological process, cellular component and molecular function. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding the high-level functions and effects of a biological system. KEGG pathway enrichment analysis was performed using the KEGG Orthology-Based Annotation System (KOBAS; http://kobas. cbi.pku.edu.cn/).
Table I. Baseline characteristics of patients with IH and control subjects.

| Characteristic          | IH (n=17) | Control (n=17) |
|-------------------------|-----------|----------------|
| Sex                     |           |                |
| Male                    | 6 (35.3)  | 6 (35.3)       |
| Female                  | 11 (64.7) | 11 (64.7)      |
| Age (months)            |           |                |
| <3                      | 12 (70.6) | 11 (64.7)      |
| ≥3                      | 5 (29.4)  | 6 (35.3)       |
| History of treatment    |           |                |
| Yes                     | 0 (0)     |                |
| No                      | 17 (100.0)|                |
| Location of IH          |           |                |
| Head, face, neck        | 6 (35.3)  |                |
| Extremity               | 3 (17.6)  |                |
| Trunk                   | 6 (35.3)  |                |
| Multiple sites          | 2 (11.8)  |                |

Values are expressed as n (%). IH, infantile hemangioma.

Statistical analysis. All data were expressed as the mean ± standard error of the mean or n (%) and analyzed using the Student's t-test with SPSS 22.0 software (IBM Corp.). GraphPad Prism 8 (GraphPad Software, Inc.) and Microsoft Excel 2019 (Microsoft Corp.) were used for other statistical analyses. P<0.05 was considered to indicate statistical significance.

Results

Demographic characteristics of patients and volunteers. In the present study, 17 patients with IH (6 males and 11 females) and 17 age-matched healthy volunteers (6 males and 11 females) were collected. Among the patients with IH, 12 cases were <3 months old and 5 cases were ≥3 months old. Among the volunteers, 11 cases were <3 months old and 6 were ≥3 months old. The sex and age distribution of patients and volunteers were not statistically different.

Differentially expressed circRNAs detected using microarray analysis. A microarray analysis of 13,617 circRNAs in the plasma of patients with IH and healthy controls was performed. Relevant data were uploaded to the Gene Expression Omnibus (GEO) database (accession no. GSE162905). A box plot was used to indicate the intensity values of the six samples after normalization (Fig. 1A). The scatter plot (Fig. 1B) and volcano plot (Fig. 1C) revealed differences in circRNA expression between the test and control groups. Finally, data analysis and screening based on FC>1.5 and P<0.05 revealed 72 upregulated and 56 downregulated circRNAs. Among the upregulated circRNAs, 48 were exonic, 15 were intronic, 5 were sense overlapping, 3 were intergenic and 1 was antisense. The downregulated circRNAs included 51 exonic, 2 intronic and 3 sense overlapping circRNAs. In addition, the distribution of these circRNAs on chromosomes was summarized, indicating that they were distributed on each chromosome. The distinct differential expression of these 128 circRNAs was visualized by hierarchical clustering in the heat map (Fig. 1D). These results indicated that the present differential expression profile was reliable.

MiRNA prediction and bioinformatics analysis of circRNAs. Based on the results of RT-qPCR, the miRNA response elements of the circRNAs were predicted using the Arraystar miRNA target prediction software. To further investigate the functions of target genes, GO and KEGG analyses of the differentially expressed circRNAs were performed using DAVID and KOBAS. Based on the RT-qPCR results, three downregulated circRNAs (hsa_circRNA_101566, hsa_circRNA_103546 and hsa_circRNA_103573) were significantly differentially expressed (Fig. 2A), consistent with the microarray results. However, no upregulated circRNAs were detected. Analysis of another seven samples revealed similar expression levels or no significant differences between patients with IH and healthy children. The three circRNAs were further verified using the remaining 14 sample pairs and significantly different expression levels were detected (Fig. 2B).

Construction of the circRNA-miRNA-mRNA network. To further evaluate the relationship between the confirmed differentially expressed circRNAs and the corresponding miRNAs and to investigate the mechanisms underlying IH development, Cytoscape was used to illustrate the hsa_circRNA_101566/hsa_circRNA_103546/hsa_circRNA_103573-miRNA-mRNA network (Fig. 4). The number of genes targeted by hsa_circRNA_101566 was the highest among all circRNAs evaluated (Fig. 4). As the amount of raw data was large, miRNAs related to angiogenesis and partially related genes were selected (referring to the increase of other tumors' angiogenesis while promoting the growth of other tumors, so its influence on blood vessels may be indirectly regulated), including hsa_miR_765,


Discussion

To the best of our knowledge, the present study was the first study to detect and profile circRNAs in the plasma of patients with IH. The results of the present study revealed that the downregulated circRNAs, namely hsa_circRNA_101566,
hsa_circRNA_103546 and hsa_circRNA_103573, targeted at least 25 different miRNAs. In recent years, the role of circRNAs in vascular diseases has been reported (21,22) and certain miRNAs have been confirmed to be involved in the pathogenesis of IH (23-25). In the present study, three pairs of plasma specimens were first analyzed using a microarray. A total of 72 upregulated and 56 downregulated circRNAs (FC≥1.5, P<0.05) were identified. Previous studies have analyzed circRNA expression in IH tumor tissues using microarray (10) and RNA-seq (26); however, circRNA expression in plasma samples from IH patients has not been previously examined. Li et al. (27), by comparing a large cohort, determined that microarray is more sensitive and efficient than RNA sequencing (RNA-seq). In addition, certain studies have suggested that plasma samples from cancer patients produce even more accurate results compared with those obtained using tissue samples to study circRNAs for diagnostic applications (28,29). In the present study, a preliminary analysis of the 128 differentially expressed circRNAs screened was performed and 6 upregulated and 4 downregulated circRNAs...
were selected for validation using RT-qPCR, including the 3 downregulated circRNAs hsa_circRNA_101566, hsa_circRNA_103546 and hsa_circRNA_103573. The results were consistent with the microarray results. Studies on circRNAs involved in IH were first performed by our group in 2017 (10), in which microarray analysis detected 234 upregulated and 374 downregulated circRNAs from four pairs of tumor tissue. Subsequently, Li et al (26) identified 124 upregulated and 125 downregulated circRNAs using RNA-seq analysis of three pairs of tumor tissue. However, the circRNAs validated by the two groups are not the same and exhibited less overlap in the experimental results. Of the circRNAs screened for statistically significant differences in the present study, only one (hsa_circRNA_104310) was mentioned in the study by Fu et al (10). The reasons for this were further analyzed. First, as only three or four sample pairs were used for the analysis in the three studies, this led to sample errors of the microarray or RNA-seq results. Furthermore, the circRNAs validated by Li et al (26) were randomly selected and it was not based on any specific strategy to select the most relevant circRNAs in their methods. Generally, it is best to choose the most distinct circRNAs or the circRNAs that may serve an important role in the regulatory network. Finally, while the present study was the first to analyze circRNAs in plasma from pediatric patients with IH, previous studies have analyzed circRNAs in tumor samples. There is likely a difference in circRNA expression...
between plasma and tumor tissue and this difference should be further explored. These results suggest that the use of different analytical methods and different samples may yield different results and that the development of IH may involve different regulatory pathways in tumor tissue and plasma; these results are consistent with the cell- and tissue-specific expression of circRNAs (30). Therefore, for circRNA investigations in IH, large samples and multicenter studies are required. In addition, the lack of comparison of tumor and plasma samples from the same patient is also a limitation of the present study. In the future, both tumor and plasma specimens from patients will be collected for validation to obtain more convincing results.

A previous study indicated that hsa_circRNA_101566 expression was significantly upregulated in IHs, suggesting that this miRNA is important in IHs (13), whereas bioinformatics analysis (using TargetScan and miRanda) suggested that hsa_circRNA_101566 and hsa_circRNA_103546 are ‘miRNA sponges’ of hsa_miR_520c. Therefore, targeting of hsa_miR_520c by hsa_circRNA_101566 and hsa_circRNA_103546 may be involved in IH development. Furthermore, certain circRNA-targeted miRNAs (hsa_miR_765, hsa_miR_486, hsa_miR_18a, hsa_miR_10a, hsa_miR_223 and hsa_miR_522) have been indicated to participate in promoting angiogenesis or inducing tumor cell proliferation (14-20) and may be related to angiogenesis and angiogenesis-related disorders in IHs.

GO is a bioinformatics tool originally designed to unify the representation of genes and gene products of various species (31).
The ontology includes three categories: Cellular component, molecular function and biological process. GO analysis of these target genes, which indirectly regulate the three downregulated circRNAs, revealed their involvement in physiological function that are closely related to the development of IH. Yang et al. (32) demonstrated that ras homolog family member A (RhoA), indirectly controlled by hsa_circRNA_101566, is able to regulate cellular apoptosis. In the absence of RhoA, the apoptosis of human umbilical vein endothelial cells induced by TNF-α was inhibited. Conversely, Del Re et al. (33) reported that RhoA/Rho kinase activation induces a mitochondrial death pathway and cardiomyocyte apoptosis, which may be correlated with the proliferation of hemangioma-derived endothelial cells during IH proliferation. Of note, this study confirmed that TOR has an important role in the vital activity of cells, while it has been indicated that mammalian target of rapamycin (mTOR) is present in humans. Medici and Olsen (34) demonstrated that rapamycin inhibits the proliferation of hemangioma-derived endothelial cells by decreasing hypoxia-inducible factor-1 (HIF-1) expression in endothelial cells and that the mTOR-related signaling pathway may be one of the mechanisms of IH pathogenesis. Thus, rapamycin is able to inhibit angiogenesis as an mTOR inhibitor and mTOR-related signaling pathways may contribute to the pathogenesis of IH. Certain target genes of hsa_circRNA_101566 are involved in the negative regulation of mTOR signaling, suggesting that mTOR signaling is upregulated and may contribute to proliferation in IH.

KEGG is a knowledge database for systematic analysis of gene function, linking genomic information with higher-order functional information (35). KEGG analysis suggested that pathways, such as the pantothenate and CoA biosynthesis pathways and the NOD-like receptor and citrate cycle pathways, are influenced by the differential expression of the circRNAs. Succinate dehydrogenase (SDH) in the citrate cycle consists of four subunits (36), namely SDHA, SDHB, SDHC and SDHD. The downregulated hsa_circRNA_103546 is able to indirectly regulate the low expression of SDHD. Selak et al. (37) reported that the accumulation of succinate due to SDH inhibition led to activation of HIF-1α, which may also be involved in the pathogenesis of IH. In addition, hsa_circRNA_101566 indirectly regulates NOD-like receptor family member X1 (NLRX1), which may be regulated by miR-520c involved in the NOD-like receptor signaling pathway (38), whereas NLRX1 is able to inhibit NF-kB and thus indirectly inhibit vascular endothelial growth factor (VEGF) (39). It was previously suggested that VEGF contributes to IH development (40) and its downregulation weakens the inhibitory effect on VEGF, which may also be a promising finding in IH. The hypoxia theory, one of the most widely accepted theories regarding the pathogenesis of IHs, states that hypoxic stress triggers the overexpression of angiogenic factors, such as VEGF, via the HIF-1α pathway (41,42).

In support of this, the present study hypothesized that the hsa_circRNA_101566/hsa-miR-520c-5p/NLRX1 signaling axis has a role in the molecular mechanisms of IH development. However, further studies are required to determine the function of circRNAs and circRNA-miRNA-mRNA networks in IH.

In conclusion, the present study was the first, to the best of our knowledge, to detect and profile circRNAs in the plasma of patients with IH. A total of 128 differentially expressed circRNAs, among which 72 were upregulated and 56 downregulated, were screened. Furthermore, three downregulated circRNAs (hsa_circRNA_101566, hsa_circRNA_103546, and hsa_circRNA_103573) were verified using RT-qPCR.
The roles of differentially expressed circRNAs were explored using GO and KEGG pathway analyses. It was determined that hsa_circRNA_101566, which is able to regulate the mTOR signaling pathway, may be a significant regulatory molecule in IH development and that the VEGF signaling pathway may be regulated indirectly by targeting hsa_miR_520c. However, a limitation of the present study was the small number of samples used; further larger studies are required in order to determine whether these circRNAs may be used as biomarkers. Furthermore, functional verification of the roles of the relevant RNA molecules in vitro and in vivo is required. These studies may provide novel ideas for accurate diagnosis and a better understanding of the pathogenesis of IH.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/).

Authors' contributions

JB was involved with conceptualization and design of the study, study supervision and statistical analysis. ZL, YC performed experimental operations, statistical analysis, data analysis and manuscript writing. ZZ and XL were involved with acquisition of data. RH helped conceptualize design and supervise the study. JB and RH checked and approved the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate statement

This study was approved by the Ethics Committee of Shandong Provincial Hospital (Jinan, China; no. 2018-051) and performed according to the Ethical Guidelines of the Declaration of Helsinki. Written informed consent was obtained from the guardian of each subject.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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