MicroRNAs (miRNAs) are endogenous small (19-24 nt) non-coding sequences of RNA that negatively regulate gene expression at a post-transcriptional level either through translational inhibition or degradation of target mRNAs. The miRNAs have the ability to regulate many genes, pathways, and complex biological networks within cells, acting either alone or in cooperation with one another. During the past decade, over 1200 miRNAs have been identified in mice, rats, and human cells, and investigations have been done to understand their various functions in normal cell homeostasis and diseases. Recently, ongoing research described possible roles of some specific miRNAs (e.g., miR-221, miR-19a/b, miR-132 and miR-146a) in a rat model of AAA using oligonucleotide microarrays.

In the present study, we used microarrays analysis to identify differentiated expression of miRNAs in late stage of experimentally induced CA and to detect possible targets of miRNAs for Cerebral aneurysm (CA) is an important acquired cerebrovascular disease that can cause catastrophic results. MicroRNAs (miRNAs) are small non-coding RNAs, playing essential roles in modulating basic physiologic and pathological processes. Currently, evidences have been established about biologic relationship between miRNAs and abdominal aortic aneurysms. However, biologic roles of miRNAs in CA formation have not been explained yet. We employed microarray analysis to detect and compare miRNA expression profiles in late stage of CA in rat model. Twenty-six, 7-week-old male Sprague-Dawley rats underwent a CA induction procedure. The control animals (n=11) were fed a normal diet, and the experimental animals (n=26) were fed a normal diet with 1% normal saline for 3 months. Then, the rats were sacrificed, their cerebral arteries were dissected, and the five regions of aneurysmal dilation on the left posterior communicating artery were cut for miRNA microarrays analysis. Six miRNAs (miRNA-1, miRNA-223, miRNA-24-1-5p, miRNA-551b, miRNA-433, and miRNA-489) were randomly chosen for validation using real-time quantitative PCR.

Among a set of differentially expressed miRNAs, 14 miRNAs were over-expressed more than 200% and 6 miRNAs were down-expressed lower than 50% in the CA tissues. The results show that miRNAs might take part in CA formation probably by affecting multiple target genes and signaling pathways. Further investigations to identify the exact roles of these miRNAs in CA formation are required.

Key Words: Intracranial aneurysm · MicroRNAs · Cell proliferation · Apoptosis · Inflammation.
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MATERIALS AND METHODS

Induction of experimental cerebral aneurysms

The left common carotid artery and the posterior branches of both renal arteries of the 26 male 7-week-old Sprague-Dawley strain rats (200 to 300 g) were ligated to induce cerebral aneurysms. These procedures were performed with the rats under intraperitoneal zoletile anesthesia (30 mg/kg) with xylazin (10 mg/kg) and additional injections if necessary. After the operation, 1% normal saline was substituted for the drinking water to enhance the degree of hypertension.

The rats were divided into control group (n=11) receiving, no operation and normal diet and aneurysm group (n=26) receiving, ligation on the left common carotid artery and the posterior branches of both renal arteries with 1% normal saline.

Animal care and experiments in this study complied with community standards on the care and use of laboratory animals.

Tissue preparation

Three months after the induction procedure, all rats were euthanized with CO gas. Cerebral arteries were dissected and stripped from their brains under a surgical microscope. We obtained the samples from the circles of Willis in the control group and from the regions of aneurysmal dilation on left posterior communicating artery in the aneurysm group (Fig. 1).

RNA isolation

Five fusiform aneurysm samples and six control samples were homogenised with tissue lyser2 (Qiagen, Germantown, MD, USA). Trizol (Invitrogen, Carlsbad, CA, USA) was used for Total RNA extraction according to the manufacturer's instructions. Low molecular weight RNA (<200 nt) was separated from the total RNA using mirVana miRNA purification columns (Ambion, Austin, TX, USA) for microarray analysis and Q-PCR according to the manufacturer's instructions. The quality and quantity of each RNA preparation were determined using a Nanodrop ND-1000 spectrophotometer (Agilent Technologies UK Ltd, West Lothian, UK).

Expression of miRNAs

Each total RNA sample (700 ng) was labeled and hybridized using FlashTag™ Biotin HSR RNA Labeling kit (manufactured for Affymetrix, Inc., by Genisphere LLC.). Total RNA was labeled using poly A polymerase. Biotin-labeled RNA were hybridized for 16-18 hr at 45°C on Affymetrix miRNA v2.0 array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450, and then were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analyzed with RMA-DABG using normalization method.

The normalized, and log transformed intensity values were analyzed using Expression Console (Affymetrix, Inc.). Fold change filters included the requirements that the genes be present in at least 200% of controls for up-regulated miRNA and lower than 50% of controls for down-regulated miRNA.

Reverse transcription and real-time quantitative PCR

To validate the up and down fold results of miRNA arrays, we assessed the expression of six miRNAs, that were randomly selected, including three down-regulated (miRNA-551b, miRNA-433, and miRNA-489) and three up-regulated (miRNA-1, miRNA-223, and miRNA-24-1-5p) miRNAs, by real-time qPCR analysis. Total RNA was extracted from aneurysm part further proving their exact roles in the pathogenesis of CA.
using NucleoSpin RNA II (MN, Germany). Reverse transcription was carried out using mature miRNA-specific primer sets (Applied Biosystems, CA, USA) and microRNA reverse transcription kit (Applied Biosystems, CA, USA) according to the manufacturer’s instructions. The miRNA-specific Taqman-based probes were purchased from Applied Biosystems and real time PCR was performed using the 7500 Fast Real Time PCR System (Applied Biosystem, CA, USA). The fold change for each miRNA was calculated using the comparative Ct (2-DDCt) method with U6 small nuclear RNA as the endogenous control. All reactions were performed in triplicate for each sample.

**RESULTS**

**Expression profiles of miRNAs in CAs and control arteries**

The expression patterns of miRNAs were found to be significantly different between these two groups as shown in Fig. 2. Fold change filters included the requirements that the genes be present in at least 200% of controls for up-regulated miRNA and lower than 50% of controls for down-regulated miRNA (Table 1). Among a set of differentially expressed miRNAs, 14 miRNAs were significantly over-expressed and 6 miRNAs were significantly down-expressed in the CA tissues as compared to that in the arteries of the control group.

**Table 1.** The list of altered microRNA (miRNA) expression in the cerebral aneurysm tissue

| miRNA          | Fold change |
|----------------|-------------|
| **Up-expressed miRNAs** |            |
| rno-miR-147    | 6.62        |
| rno-miR-101b   | 5.88        |
| rno-miR-1      | 5.79        |
| rno-miR-21     | 5.79        |
| rno-miR-451    | 3.70        |
| rno-miR-223    | 2.82        |
| rno-miR-29c    | 2.71        |
| rno-miR-29b    | 2.70        |
| rno-miR-26b    | 2.66        |
| rno-miR-140    | 2.30        |
| rno-miR-22-5p  | 2.22        |
| rno-miR-29a    | 2.15        |
| rno-miR-24-1-5p| 2.07        |
| rno-miR-181c   | 2.07        |
| **Down-expressed miRNAs** |        |
| rno-miR-551b   | 23.74       |
| rno-miR-433    | 8.23        |
| rno-miR-489    | 7.08        |
| rno-miR-138    | 6.02        |
| rno-miR-92b    | 6.02        |
| rno-miR-181d   | 2.04        |

**Validation of the microarray analysis data by real-time qPCR**

The relative expression changes of these miRNA analyzed by real-time qPCR were consistent with the microarray analysis results, as shown in the histogram (p<0.05) in Fig. 3.

**Statistical analysis**

Statistical analysis was performed by Student’s t-test using the SPSS 13. Differences were considered statistically significant at p<0.05.

**DISCUSSION**

Among the up-regulated miRNAs validated in this study, over-expression of miR-24 negatively controlled the TGFβ signaling pathway via silencing Trb3 and induced myogenic activity by the regulation of the vascular smooth muscle cell (VSMC) phenotype switch4. Current evidences indicated that apoptosis in VSMCs was significantly increased and expression of the caspase-3 gene was up-regulated in human intracranial aneurysm8. In recent miRNAs and AAA studies, it was suggested that enhanced miR-21 expression may serve as an endogenous response to pathological aortic dilatation. Increased miR-21 expression resulted in a pro-proliferative and antiapoptotic response of VSMCs within the vessel wall, most likely in an attempt to protect the aorta from further expansion and ultimate rupture13. Over-expression of miR-22 acted as an integrator of Ca++ homeostasis and myofibrillar protein content during stress in the heart9. These studies support the idea that the interactions of these up-regulated miRNAs suppressing the apoptosis and promoting cell proliferation might modulate the cell proliferation in VSMCs of CAs against the hemodynamic stress.

The degradation of extracellular matrix (ECM) is a hallmark of a CA formation. Tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 were expressed mainly by VSMCs in the aneurysmal walls. The mRNA expression of metalloproteinase (MMP)-21 and MMP-9 dramatically increased in the late stage of experimentally induced CA. In both TIMP-1(-/-) mice and TIMP-2(-/-) mice, CA progression was enhanced with the increased enzyme activity of MMPs. Aoki et al.1 suggested that TIMP-1 and TIMP-2 have a protective role for CA progression. There is an imbalance between MMPs and TIMPs in the late stage of cerebral aneurysm formation, which may be responsible for ECM degradation leading to the progression and rupture of cerebral aneurysms. Over-expression of miR-1, miR-26a, miR-30d, miR-24, miR-29a, miR223 and miR-181c in MMP-9 knockout mice reduced PAR-1 mediated cardiomyocyte dysfunction and improve cardiac function14. In this study, over-expression of miR-1 (5.8 fold), miR-223 (2.8 fold), miR-29a (2.2 fold), miR-24-1-5p (2.1 fold) and miR-181c (2.1 fold) were identified. Our specimens were also in the late stage of experimentally induced CA as Aoki et al.1, indicating that these miRNAs may act as a protective role for CA progression.
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Aside from controlling cell proliferation, we found that expression of the miR-29 family, consisting of three mature members miR-29a, miR-29b and miR-29c, was up-regulated. The miR-29b was related to protein metabolism and the miR-29a was related to immune function[15].

The miR-23 has been revealed to be a novel regulator of macrophage activation in inflammatory response, which suppresses proinflammatory pathways and enhances the antiinflammatory response[16]. The known roles of miR-223 were compatible with the inflammatory features of CAs. Hemodynamic stress triggers vascular remodeling including VSMCs and induces infiltration of inflammatory cells, especially macrophages, into the intracranial aneurysmal walls[17]. Over-expression of miR-223 may play a protective role to vascular homeostasis and inflammation.

Among the down-regulated miRNAs validated in this study, quickly down-regulated miR-489 was detected during satellite-cell activation in muscle stem cell and it was highly expressed in quiescent satellite cells[18]. These results suggest that down-regulated miR-489 may promote the transient proliferative expansion of myogenic progenitors. Ectopic expression of miR-181d suppressed proliferation in glioma cell lines[19]. It means that down-regulated miR-181d can suppress the apoptosis and promoted the cell proliferation.

In the present study, highly down-regulated miR-551b and highly up-regulated miR-147 and miR-101b were identified but exact roles of these miRNAs have not been fully investigated until now.

We used an experimentally induced cerebral aneurysm model in rats. This model has native characteristics and is sufficiently similar to human cerebral aneurysms. In this study, altered expression of dysregulated miRNA was identified at the late phase of CA formation. Our results revealed that several miRNA are differentially expressed by comparing the miRNA profiles between CA and normal cerebral arteries in rats. Our research may present an insight into the pathogenesis for formation of CAs. In addition, we assessed the expression of six miRNAs, randomly selected, including three down-regulated and three up-regulated miRNAs, by real-time qPCR analysis. These PCR data were compatible with microarray data. Therefore, the microarray data can provide a meaningful starting point for interpreting potential roles of miRNAs in the formation of CA. Above all, we must focus on detecting the roles of miRNA in vascular remodelling, apoptosis and proliferation of VSMC and vascular inflammation associated with development of CA.

CONCLUSION

Out data indicate that miRNAs might take part in CA formation probably by affecting multiple target genes and signaling pathways. This study provides an overall view of miRNA expression profiles in experimentally induced CAs and strongly
supports the idea that miRNAs play an important role in pathological processes in vascular diseases. Further investigations are needed to identify the exact roles of these miRNAs in the formation of CA.

- Acknowledgements

This work was supported by The Catholic University of Korea Daejeon St. Mary's Hospital, Clinical research institute Grant funded by The Catholic University of Korea Daejeon St. Mary's Hospital (CMCDJ-P-2012-018).

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