Differential Expression of microRNAs in Severely Calcified Carotid Plaques

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Background: We investigated whether microRNA (miRNA) alteration is related to the presence of calcification in carotid plaques. *Methods:* We classified 10 plaques from carotid endarterectomy patients into high- and low-calcified plaques based on Agatston calcium scores. A microarray analysis for miRNA profiles was performed, with validation by a miRNA quantitative real-time polymerase chain reaction (qRT-PCR). *Results:* The miRNA microarray identified 697 probes; 657 of them were downregulated. We selected the genes that satisfied total gene signal (TGS) >50, |Log2 ratio| > 1 and ≥1 of the following: (1) false discovery rate (FDR) <.05 in the comparison of mean values of logarithmic transformed signals between the groups; (2) .05 ≤ FDR < .1 and showing either high or median for context score+ in miRSearch among the 72 carefully selected genes related to angiogenesis or calcification; and (3) FDR < .1 in the comparison of 10 individual sets of high- and low-calcified plaques. The expression of miRNA validated by qRT-PCR revealed a significant downregulation of hsa-miR-4530, hsa-miR133b, and hsa-miR-1-3p. A Spearman's rank correlation analysis revealed that the logarithmic TGSs for the microarray of hsa-miR-4530 and hsa-miR-133b were significantly inversely correlated with the carotid plaques' calcium scores, and the delta Cq values for the qRT-PCR showed a direct association. *Conclusions:* In high-calcified carotid plaques, a specific profile for miRNA may be identified, and the expressions of hsa-miR-4530 and hsa-miR-133b had inverse correlations with the calcium score in the plaques, suggesting that miRNAs may play a modulating role in calcified plaques and plaque stability. **Key Words:** microRNA—carotid plaque—calcification—microarray analysis—angiogenesis.
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Introduction

Both the precise mechanisms underlying the molecular processes of calcification and the clinical roles of calcification in the carotid wall have not been well elucidated, although these mechanisms are reported to influence symptomatology (as in cardiovascular events that involve a coronary artery).[1] We previously demonstrated the upregulation of Angiopoietin-like 4 (ANGPTL4) mRNA and proteins as well as the downregulation of fibroblast growth factor receptor 2 (FGFR2) in highly calcified carotid plaques, and our findings suggested that an antiangiogenic modulating function by these molecules may contribute to the stability of carotid plaques.[2]

MicroRNAs (miRNAs) are short (18-24 nucleotides in length) noncoding RNAs. They regulate gene expression post-transcriptionally by either degradation or translational repression, binding to complementary coding sequences of many different target mRNAs.[3] An individual miRNA may regulate multiple target mRNAs, and a single mRNA can be controlled by several miRNAs; thus, almost one third of all human genomes are thought to be regulated by miRNAs.[4] As for vascular calcification, miR-204, miR-135a*, miR-762, miR-714, miR-712*, miR-29b, miR-133b, and miR-211 have been reported to be involved in the calcification of mice vascular smooth muscle cells (VSMCs) or aortic media.[5-7] However, few studies have focused on the impact of the miRNA expression profile on vascular calcification by using the human carotid artery.

As mentioned above, we observed that an antiangiogenic effect of dysregulated transcripts may contribute to the stability of highly calcified plaques.[2] To date, hsa-miR-221/222, miR-328, miR-92a, and miR-214 have been reported as antiangiogenic miRNAs in atherosclerosis studies, and miR-126, miR-130a, miR-210, miR296, miR-17-92, let-7, and miR378 were described as angiogenic.[8] Accordingly, modulation by miRNAs targeting angiogenesis or growth factors may also play an important role in calcified stable plaques.

In this study, we examined whether mature miRNAs are associated with the presence and degree of calcification in human carotid plaques by performing a miRNA microarray and miRNA quantitative real-time polymerase chain reaction (qRT-PCR). We also investigated whether any miRNA influences the previously verified ANGPTL4, FGFR2, or other genes related to calcification, osteogenesis, angiogenesis, and growth factors in highly calcified plaques by conducting a data-mining study.

Materials and Methods

Patients and Specimens

Carotid plaques from 10 endarterectomy patients were investigated (Table 1). Half of the plaques were highly calcified plaques (H1-5), which showed a mean calcium score of 1601.6 ± 886.9, and the rest of the plaques were low calcified (L1-5) with a mean calcium score of 95.2 ± 96.0. The characteristics of the plaques other than calcification (i.e., lipid core, fibrous tissue, and hemorrhage) were observed by examining hematoxylin–eosin stained specimens, and we stratified each finding into three degrees or none. Macroscopic hemorrhages were relatively more frequently found in the low-calcified plaques compared with the high-calcified plaques. No remarkable difference was found between the high- and low-calcified plaques concerning clinical data including the degrees of stenoses ($77.4 \pm 24.1\%$ versus $67.8 \pm 18.6\%$, $P = .50$) except for the patients' rates of hypertension and smoking habit.

Table 1. *The characteristics of the plaques and the patients' clinical data*

Plaque	Ca score	Lipid core	Fibrous tissue	Hemorrhage	Age	Gender	Stenosis (%)	Symptom	Hypertension	Diabetes mellitus	Dyslipidemia	IHD w/PCI	Renal malfunction	Smoking	Antiplatelet	Anticoagulant
H1	912.0	+++	+	−	63	M	52.0	S	+	−	+	−	+	−	−	−
H2	958.9	++	−	−	78	F	95.0	S	+	−	−	−	−	−	+	−
H3	1026.2	−	+	−	74	M	95.0	S	+	+	+	−	−	−	+	−
H4	2324.3	−	++	−	63	M	50.0	S	+	+	+	−	−	−	+	+
H5	2786.6	−	+	+	78	F	95.0	A	+	−	−	−	−	−	+	−
L1	0	+	+	−	62	F	50.0	S	+	−	+	−	−	+	+	−
L2	15.1	++	+	−	57	F	85.0	A	−	−	−	−	−	−	+	−
L3	94.8	−	+	−	76	M	65.0	A	+	−	−	−	−	−	+	−
L4	128.9	+	+	−	69	M	89.0	S	−	−	−	−	−	+	+	−
L5	237.4	+++	+	+	65	M	50.0	S	+	+	+	−	−	−	+	−

Abbreviations: A, asymptomatic; F, female; H1-5, high-calcified plaques; IHD, ischemic heart disease; L1-5, low-calcified plaques; M, male; PCI, percutaneous coronary intervention; S, symptomatic.