Acute Loss of miR-221 and miR-222 in the Atherosclerotic Plaque Shoulder Accompanies Plaque Rupture

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Background and Purpose—Atherosclerotic plaque vulnerability is accompanied by changes in the molecular and cellular function in the plaque shoulder, including a decrease in vascular smooth muscle cell proliferation. We aimed to determine whether the expression of 3 miRNAs that regulate vascular smooth muscle cell proliferation (miR-145, miR-221, and miR-222) is altered with plaque rupture, suggesting a role in regulating plaque stability.

Methods—miRNAs were measured in the plaque shoulder of carotid plaques obtained from patients undergoing carotid endarterectomy (CEA) for 3 distinct clinical scenarios: (1) patients without previous neurological events but high-grade carotid stenosis (asymptomatic), (2) patients with an acute neurological event within 5 days of the CEA (urgent), and (3) patients undergoing CEA >5 days after a neurological event (symptomatic).

Results—Mean time from plaque rupture event to CEA was 2.4 days in the urgent group. The urgent group exhibited a significant decrease in miR-221 and miR-222 expression in the plaque shoulder, whereas no significant differences were seen in miR-145 across the 3 groups. Regression analysis demonstrated a significant correlation between time from the neurological event to CEA and increasing miR-221 and miR-222, but not miR-145. mRNA encoding p27Kip1, a target of miR-221 and miR-222 that inhibits vascular smooth muscle cell proliferation, was increased in the urgent group.

Conclusions—Atherosclerotic plaque rupture is accompanied by a loss of miR-221 and miR-222 and an increase in p27Kip1 mRNA expression in the plaque shoulder, suggesting an association between these miRNAs and atherosclerotic plaque stability. (Stroke. 2015;46:3285-3287. DOI: 10.1161/STROKEAHA.115.010567.)

Key Words: atherosclerosis ■ carotid stenosis ■ endarterectomy, carotid ■ microRNAs ■ muscle, smooth, vascular

Carotid plaque rupture leads to acute neurological symptoms, and, similarly, coronary plaque atheroembolization leads to acute coronary syndrome. Loss of vascular smooth muscle cells (VSMCs) in the plaque shoulder region of the fibrous cap has been implicated in plaque rupture. MicroRNA-221 and microRNA-222 (miR-221/miR-222) are short noncoding RNAs that inhibit the expression of the cyclin-dependent kinase inhibitor, p27Kip1, promoting VSMC proliferation and intimal thickening. In contrast, miR-145 promotes VSMC differentiation, as well as decreased intimal thickening and atherosclerotic plaque formation in animal models.

The present study aimed to investigate the role of expression of these miRNAs in atherosclerotic plaque rupture. We measured miR-221/miR-222 and miR-145 in carotid plaque shoulder segments from patients undergoing carotid endarterectomy (CEA) and stratified the data according to the time between the CEA and the neurological event. Here, we report that miR-221/miR-222 expression is significantly decreased acutely after plaque rupture and exhibits a rapid return to pre-rupture levels.

Methods
Carotid plaque specimens and pertinent medical histories were obtained from 76 patients undergoing CEA in the Department of Surgery at the Ochsner Clinic with no significant differences in age, sex, lipid profile, smoking, or the presence of diabetes mellitus between the groups (Table). Similar to our previous finding, there was a significant increase in miR-221/miR-222 expression in diabetic versus nondiabetic patients (P<0.01; Figure I in the online-only Data Supplement). Patients were stratified into 3 groups: without previous neurological events (asymptomatic, n=31), with a previous neurological event within 5 days of the CEA (urgent, n=25), and undergoing CEA >5 days after a neurological event (symptomatic, n=20). miRNAs and mRNAs of interest were measured in the plaque shoulder by real-time polymerase chain reaction using the primers listed in Table 1 in the online-only Data Supplement. A more detailed Methods is available in the online-only Data Supplement.
**Results**

The urgent group exhibited a significant decrease in miR-221/miR-222, but not in miR-145, when compared with the asymptomatic and symptomatic groups (Figure [A]). Linear regression analysis demonstrated a direct correlation between expression of miR-221/miR-222 and increasing time between carotid plaque rupture/acute neurological symptom onset and the time when CEA occurred ($R^2=0.44; P<0.001$ for miR-221 and $R^2=0.45; P<0.001$; Figure [B]). This correlation is not seen with miR-145 ($R^2=0.004; P=0.85$).

Levels of the mRNA encoding the miR-221/miR-222 target, p27Kip1, were increased acutely post-plaque rupture (urgent group; Figure [C]). Levels of 2 other targets of miR-221/222 that are involved in neovascularization,$^8,9$ c-Kit and the signal transducer and activator of transcription 5A, remained unchanged across the groups, suggesting neovascularization is not altered by the changes in expression of miR-221/miR-222 that occur with rupture. These data suggest a role for loss of miR-221/miR-222 inhibition of p27Kip1 in the thinning of the fibrous cap that promotes plaque instability and rupture.

**Discussion**

This is the first demonstration that miR-221/miR-222 expression in the plaque shoulder is decreased acutely after plaque rupture. The loss of miR-221/222 was accompanied by an increase in the mRNA encoding its target, p27Kip1. Loss of p27Kip1 through increased miR-221/miR-222 expression results in increased intimal thickening in animal models of vascular injury.$^1$ VSMCs isolated from advanced atherosclerotic plaques exhibit lower proliferation rates.$^{10,11}$ Similarly, VSMCs isolated from CEA specimens obtained from asymptomatic patients exhibit higher proliferative responses and lower p27Kip1 levels than those obtained from symptomatic patients.$^{12}$ Our data support a role for miR-221/miR-222 in the intimal thickening associated with plaque development, and that loss of miR-221/222 may underlie the reduced VSMC proliferation associated with fibrous cap thinning and plaque rupture. Downregulation of miR-221/miR-222 has also been implicated in the regulation of plaque neovascularization and monocyte differentiation to macrophages.$^9,13$ Although we focused our studies on the effects of loss of miR-221/miR-222...

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**Table. Characteristics of Patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n=76)</th>
<th>Asymptomatic (n=31)</th>
<th>Urgent (n=25)</th>
<th>Symptomatic (n=20)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66.8±2.0</td>
<td>68.2±2.9</td>
<td>66.8±2.6</td>
<td>64.5±5.3</td>
<td>0.28</td>
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<tr>
<td>Male sex</td>
<td>50 (66)</td>
<td>19 (61)</td>
<td>18 (72)</td>
<td>13 (65)</td>
<td>0.70</td>
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<tr>
<td>Body mass index, kg/m²</td>
<td>27.1±0.8</td>
<td>28.5±1.1</td>
<td>26.4±1.5</td>
<td>25.9±1.6</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>175.9±6.7</td>
<td>166.1±11.4</td>
<td>184.5±9.1</td>
<td>175.0±17.6</td>
<td>0.42</td>
</tr>
<tr>
<td>High-density lipoprotein, mg/dL</td>
<td>43.0±1.6</td>
<td>44.2±2.3</td>
<td>40.0±2.3</td>
<td>46.8±4.2</td>
<td>0.22</td>
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<tr>
<td>Low-density lipoprotein, mg/dL</td>
<td>102.2±5.2</td>
<td>96.7±9.6</td>
<td>108.8±6.5</td>
<td>98.2±12.9</td>
<td>0.56</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>134.2±8.8</td>
<td>124.6±11.6</td>
<td>153.6±16.6</td>
<td>112.2±11.8</td>
<td>0.14</td>
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<tr>
<td>Serum creatinine, mg/dL</td>
<td>1.1±0.1</td>
<td>1.0±0.0</td>
<td>1.0±0.1</td>
<td>1.3±0.2</td>
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<tr>
<td>Diabetes mellitus</td>
<td>13 (17)</td>
<td>5 (16)</td>
<td>5 (20)</td>
<td>3 (15)</td>
<td>0.89</td>
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<td>Smoker</td>
<td>21 (28)</td>
<td>9 (29)</td>
<td>8 (32)</td>
<td>4 (20)</td>
<td>0.81</td>
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<tr>
<td>Time to CEA, d</td>
<td>...</td>
<td>2.4±0.4</td>
<td>38±8</td>
<td>&lt;0.001</td>
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</table>

CEA indicates carotid endarterectomy.

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**Figure.** Expression of miRNA and their targets in the carotid plaque shoulder. **A**, Quantification of miR-145 and miR-221/miR-222 in the plaque shoulder of patients with no previous neurological event (asymptomatic), a neurological event within 5 d before the carotid endarterectomy (CEA; urgent), and a neurological event >5 d before the CEA (symptomatic). **B**, X–Y scatter plot of fold changes in miR-221/miR-222 expression vs time between neurological event and CEA for patients undergoing CEA within 12 d of the cerebrovascular event. The solid and dashed lines represent a linear regression fit to the miR-221 and miR-222 data, respectively. **C**, Quantification of p27Kip1, signal transducer and activator of transcription 5A (STAT5A), and c-Kit in the plaque shoulder of asymptomatic and urgent patients. †P<0.05.
in VSMCs, additional studies examining the role of miR-221/miR-222 in regulating plaque neovascularization and macrophage accumulation are, therefore, warranted.

By focusing on carotid plaques obtained from patients undergoing urgent CEAs, we were able to identify modulations in miRNA expression that occur acutely with plaque rupture, but would not be detected in the standard symptomatic patient. Namely, that miR-221/miR-222 expression is reduced in the carotid plaque shoulder at the time of rupture and then returns to prerupture levels within 2 weeks. It is not possible, however, to determine whether the decrease in miR-221/miR-222 occurs before or as a result of plaque rupture. These data highlight the unique value of obtaining samples immediately after an acute neurological event when examining the mechanism underlying plaque rupture and suggest a link between miR-221/miR-222 and cell proliferation in this region.

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Disclosures
None.

References
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The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/11/3285

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/10/08/STROKEAHA.115.010567.DC1
SUPPLEMENTAL MATERIAL

Detailed Methods

ICA collection and Patient Stratification

Carotid artery atherosclerotic plaque specimens and pertinent medical histories were obtained from 76 patients undergoing CEA in the Department of Surgery, Section of Vascular/Endovascular Surgery at the Ochsner Clinic. The Ochsner Internal Review Board approved the protocol and informed consent was obtained from all participants. Plaques were cross-sectioned into ~1cm pieces and the plaque shoulder was isolated using gross dissection, snap-frozen, and stored at -80°C until RNA isolation. The patients were stratified according to their neurologic presenting symptomatology; a stroke neurologist independently assessed all acutely symptomatic carotid patients. The mean NIH Stroke Score for the acutely symptomatic patients presenting with a stroke was 3.3 ± 0.7. Patients without prior neurologic events but high-grade (>80%) carotid stenosis undergoing a prophylactic CEA were termed “asymptomatic” (n = 31). Patients with a prior neurologic event, including transient monocular vision loss, a transient cerebral ischemic event or stroke, indicated an acute plaque rupture event and were grouped according to time between the ischemic event and the CEA. Patients undergoing CEA within 5 days of rupture were termed “urgent” (n = 25) and those undergoing CEA greater than 5 days post rupture were termed “symptomatic” (n = 20). The mean times to treat in the urgent and symptomatic groups were 2.4 ± 0.4 and 38 ± 8 days, respectively.

RNA Isolation and Analysis

For each carotid plaque, the cross-section with the greatest plaque burden was identified and the circumferential boundary region of the plaque (plaque shoulder) was dissected away. Total RNA was isolated from these specimens using the miRNeasy mini kit (Qiagen Inc., Valencia, CA) with minor modifications. miRNA were measured using the miScript II RT Kit coupled with the miScript SYBR Green PCR Kit (Qiagen). STAT5A, c-Kit, and p27Kip1 were quantified using the One-step Quantitect SYBR Green PCR Kit (Qiagen). U6 snRNA and Hypoxanthine Phosphoribosyltransferase (Hprt) were used as loading controls for the miRNA assays and mRNA assays, respectively. The catalog numbers for the individual PCR assays are listed in the Supplementary Table I. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The differences observed in miR-221/222 remained significant after adjusting for the individual performing the isolation.

Statistics

Data is expressed as the mean +/- standard error of the mean. Statistical analysis between three groups was performed using ANOVA coupled with Tukey's HSD test. For comparisons between two groups, Students t-test was used. $X^2$ analysis was used to compare categorical variables across groups. All analyses were performed using SPSS v19.0 (IBM).
Supplementary Table I

Supplemental Table: Primer Assays

<table>
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**Supplementary Figure I.** Expression of miRNA in the carotid plaque shoulder of diabetic and non-diabetic patients. † indicates p < 0.05.