A Unique MicroRNA Signature Associated With Plaque Instability in Humans

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Background and Purpose—Atherosclerotic plaque rupture is considered the most important mechanism that underlies the onset of stroke, myocardial infarction, and sudden death. Several evidences demonstrated the pivotal role of inflammatory processes in plaque destabilization. MicroRNAs (miRNAs) are small endogenous RNAs and represent a new important class of gene regulators. Nevertheless, no data exist about the expression profile of miRNAs in atherosclerotic plaques. Thus, the aim of this study was to investigate the expression level of miRNAs in human plaques and to correlate it with clinical features of plaque destabilization.

Methods—Two separate groups of plaques were collected from patients who underwent carotid endarterectomy in Chieti (n=15) and Ancona (n=38) Hospitals. All the plaques were subdivided in symptomatic (n=22) and asymptomatic (n=31) according to the presence/absence of stroke.

Results—First, on the plaques collected at Chieti Hospital, we performed large-scale analysis of miRNA expression. Between the 41 miRNAs examined, we discovered profound differences in the expression of 5 miRNAs (miRNA-100, miRNA-127, miRNA-145, miRNA-133a, and miRNA-133b) in symptomatic versus asymptomatic plaques. Remarkably, when we repeated the analysis on the Ancona plaque subset, all these 5 miRNAs confirmed to be significantly more expressed in the symptomatic plaques. Finally, in vitro experiments on endothelial cells transfected with miRNA-145 and miRNA-133a confirmed the importance of these miRNAs in the modulation of stroke-related proteins.

Conclusions—These results are the first to report alterations in the expression of specific miRNAs in human atherosclerotic plaques and suggest that miRNAs may have an important role in regulating the evolution of atherosclerotic plaque toward instability and rupture. Furthermore, by identifying the specific miRNA signature for stroke now, we are able to use computer algorithms to identify previously unrecognized molecular targets.

Key Words: atherosclerosis • inflammation • microRNA • vulnerable plaque

Atherosclerotic plaque rupture is considered the most important mechanism that underlies the onset of acute ischemic syndromes, including stroke, unstable angina, acute myocardial infarction, and sudden death. Although the pathophysiology of plaque rupture is not completely understood, it is now well accepted that lesion vulnerability is more correlated with its composition rather than its size. In this regard, much evidence demonstrated the pivotal role of inflammatory processes, neangiogenesis, and apoptosis in plaque destabilization: unstable lesions, typically characterized by a large lipid core underlying a thin fibrous cap, contain a greater inflammatory infiltration, a higher number of neovessels, and a greater apoptotic status compared with stable plaques. Thus, the identification of the mechanism(s) underlying the higher inflammatory and apoptotic reaction in atherosclerotic plaque could therefore promote the development of novel intervention strategies.

In this light, developmental biologists working on the primitive earthworm Caenorhabditis elegans discovered a novel regulatory mechanism involving short pieces of RNA (microRNA [miRNA]) for which they were awarded the 2006 Nobel Prize in Physiology/Medicine. miRNAs are endogenous, noncoding, single-stranded RNAs of approximately 20 nucleotides and represent a new class of gene regulators. miRNAs are initially transcribed by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. The pri-miRNAs are then modified by RNA polymerase II in the nucleus to form large pri-miRNA transcripts.
regulate gene expression by blocking protein translation (binding to 3’UTR of mRNA with imperfect complementarity) or inducing targeted mRNA cleavage (binding to mRNA with perfect complementarity). Several miRNAs are highly conserved, thus reflecting their great value for organ homeostasis. At present, >400 miRNAs have been sequenced in human, and 1000 miRNA genes are estimated to exist in the human genome (miRBase: http://microrna.sanger.ac.uk/). As a group, miRNAs are estimated to regulate approximately 30% genes of the human genome, and therefore miRNAs represent important endogenous regulators for gene expression and actively participate in all the main biological process, ranging from cell growth, differentiation, and apoptosis.

Nevertheless, despite the fact that miRNA activity is considered critical in the regular cell growth and physiology, and accordingly, dysregulation of miRNA function may be responsible for several human diseases, the biological roles of only few miRNAs have been clarified to date. In particular, the only research area in which the role of miRNAs has been largely elucidated is cancer, starting from the assumption that cell dedifferentiation, growth, and apoptosis are essential events in the development of cancer. Indeed, both basic and clinical studies have definitely demonstrated that miRNAs are aberrantly expressed in various hematopoietic and solid tumors.

In contrast, although miRNAs are clearly expressed in the cardiovascular system, the role of miRNAs in vascular diseases is almost completely unknown. Recently, Ji and coworkers have recognized an essential role of miRNAs in vascular neointimal lesion formation by demonstrating that modulation of an aberrantly overexpressed miRNA, miR-21, had a significant negative effect on neointimal lesion formation. However, at this time, no data exist regarding the expression profile of miRNAs in established atherosclerotic plaques in humans and if expression of specific miRNA signature(s) may contribute to plaque evolution toward an unstable phenotype. Thus, the aim of this study was to investigate the expression of miRNAs in human plaques and to correlate it with clinical features of plaque destabilization.

**Methods**

**Patients**

Two independent cohorts of atherosclerotic plaques were collected in 2 different Italian hospitals, Chieti (n=15: I series) and Ancona (n=38: II series), from patients who underwent carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis. Patients’ characteristics are described in Table 1. The degree of luminal narrowing was determined by repeated Doppler echography and intra-arterial cerebral angiography using the criteria of the North American Symptomatic Carotid Endarterectomy Trial. Written informed consent for biological studies was obtained from all patients analyzed. The plaques were subdivided into 2 groups according to the presence or absence of clinically related stroke (first group: symptomatic plaques, n=22; second group: asymptomatic plaques, n=31). The plaques were excised, appropriately dissected, preserved in RNA later (Chieti group) or flash-frozen in liquid nitrogen (Ancona group), and finally stored at −80°C for subsequent molecular analyses.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chieti Cohort</th>
<th>Ancona Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td></td>
<td>(n=15)</td>
<td>(n=38)</td>
</tr>
<tr>
<td>Age, y</td>
<td>76±3</td>
<td>75±4</td>
</tr>
<tr>
<td>Male/female</td>
<td>7/0</td>
<td>8/0</td>
</tr>
<tr>
<td>Recent history of stroke</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Recent history of IH</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>NSAID or ASA treatment</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Stenosis severity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean± SD</td>
<td>76±8</td>
<td>75±4</td>
</tr>
<tr>
<td>Range</td>
<td>70–90</td>
<td>70–90</td>
</tr>
</tbody>
</table>

IH indicates ischemic heart disease; NSAID, nonsteroidal anti-inflammatory drug; ASA, acetylsalicylic acid; SD, standard deviation.

**RNA Extraction**

The RNA was extracted from atherosclerotic plaques using the mirVana miRNA Isolation Kit (Ambion, Austin, TX), which allows the recovery of small RNAs (<200 bases), including miRNAs, according to the manufacturer’s instructions. All RNA samples were subjected to DNase I digestion to prevent genomic DNA contamination and then stored at −80°C until use.

**miRNA Expression by Quantitative Real-Time Polymerase Chain Reaction Assay**

To identify the miRNA target genes, we used different gene prediction tool available online such as Targetscan (www.targetscan.org), miRanda (www.microrna.org), and TarBase (http://dianna.csbl.ece.unt.edu/tarbase). After that, the following 41 miRNAs were selected: mir100, mir103, mir104, mir105, mir106a, mir107, mir122a, mir124a, mir124b, mir125a, mir125b, mir126, mir127, mir128a, mir128b, mir129, mir130a, mir130b, mir132, mir133a, mir133b, mir134, mir135a, mir135b, mir137, mir138, mir139, mir140, mir141, mir142 3p, mir142 5p, mir144, mir145, mir146, mir147, mir148a, mir149, mir150, mir151, mir152, and mir16. We selected these miRNAs according to the involvement of their target genes in the pathophysiology of atherosclerotic plaque growth and instability. In particular, target genes of these miRNAs are recognized to be involved in extracellular collagen turnover (matrix metalloproteinase-9); inflammatory reactions (CD40 and BCL6); smooth muscle cells dedifferentiation and proliferation (KLF-15, platelet-derived growth factors, and insulin growth factor-1 pathway); and eicosanoid metabolism (PTGIS, CDC4-2, and RhoA).

A complete list of these regulated genes is depicted in Table 2.

| miRNA | Primers/probe | Applied Biosystems | Foster City, CA | Used for semi-quantitative determination of the expression level in both asymptomatic and symptomatic plaques. First, 100 ng of total RNA was reverse-transcribed using miR-specific stem-loop reverse transcription primers (50 nmol/L) and reagents purchased from the Applied Biosystems High-Capacity cDNA Archive Kit (1× reverse transcription buffer, 0.25 μmol/L each of dNTPs, 3.33 U/μL Multiscribe reverse transcriptase, 0.25 U/μL RNase inhibitor) in the GeneAmp 9700 polymerase chain reaction (PCR) system (Applied Biosystems). The reactions were performed using the ABI PRISM 7000 Sequence Detector. The reactions were performed using the ABI PRISM 7000 Sequence Detector.
Table 2. Target Genes of Selected MicroRNAs

<table>
<thead>
<tr>
<th>hsa-microRNA</th>
<th>Target Genes</th>
<th>Source</th>
<th>Role in Atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-145</td>
<td>CD40</td>
<td>Targetscan</td>
<td>Modulation of inflammation (Schönbeck et al, 2001)</td>
</tr>
<tr>
<td>mir-145</td>
<td>ABCA1 (ATP-binding cassette, subfamily A [ABCA1], member 1)</td>
<td>Targetscan</td>
<td>Reverse cholesterol transport (Soumian et al, 2005)</td>
</tr>
<tr>
<td>mir-145</td>
<td>IRS1 (insulin receptor substrate 1)</td>
<td>TarBase/Shi et al, 2007</td>
<td>Member of IGF-1 pathway, beneficial effect on plaque stabilization (Okura et al, 2001)</td>
</tr>
<tr>
<td>mir-145</td>
<td>PDGF (platelet-derived growth factor D)</td>
<td>Targetscan/Cheng et al, 2009</td>
<td>Modulates VSMCs differentiation (Swartz et al, 2007)</td>
</tr>
<tr>
<td>mir-145</td>
<td>PAI-1 (plasminogen activator inhibitor-1, SERPINE 1)</td>
<td>Targetscan</td>
<td>Modulation of atherothrombosis (Lupu et al, 1993)</td>
</tr>
<tr>
<td>mir-145</td>
<td>MYOCD (myocardin)</td>
<td>Parmacek, 2009; Cordes et al, 2009</td>
<td>Modulates VSMCs differentiation (Cheng et al, 2009)</td>
</tr>
<tr>
<td>mir-145</td>
<td>IGF1R (insulin growth factor 1 receptor)</td>
<td>La Rocca et al, 2009</td>
<td>Member of IGF-1 pathway, beneficial effect on plaque stabilization (Li et al, 2003)</td>
</tr>
<tr>
<td>mir-145</td>
<td>SRF (serum response factor)</td>
<td>Xin et al, 2009</td>
<td>Modulates VSMCs differentiation</td>
</tr>
<tr>
<td>mir-133a,b</td>
<td>MPP-9 (matrix metallopeptidase 9/gelatinase B, 92 kDa gelatinase)</td>
<td>Targetsan/Mishra et al, 2009</td>
<td>Degradation of extracellular matrix (Cipollone et al, 2001)</td>
</tr>
<tr>
<td>mir-133a,b</td>
<td>CASP9 (caspase 9, apoptosis-related cysteine peptidase)</td>
<td>Xu et al, 2007</td>
<td>Modulation of apoptosis (Yao et al, 2001)</td>
</tr>
<tr>
<td>mir-133a,b</td>
<td>KLF15 (Kruppel-like factor 15)</td>
<td>Horie et al, 2009</td>
<td>Modulation of VSMCs migration and proliferation (Lu et al, 2010)</td>
</tr>
<tr>
<td>mir-133a,b</td>
<td>MMP15 (matrix metallopeptidase 15)</td>
<td>Targetsan</td>
<td>ECM degradation</td>
</tr>
<tr>
<td>mir-133a</td>
<td>PTGIS (prostacyclin synthase)</td>
<td>Miranda</td>
<td>inhibitor of leukocyte adhesion and platelet aggregation (Cheng et al, 2002)</td>
</tr>
<tr>
<td>mir-133a</td>
<td>CDC42</td>
<td>Carè et al, 2007</td>
<td>Modulates COX-2 and MPP-9 expression (Xue et al, 2008)</td>
</tr>
<tr>
<td>mir-133a</td>
<td>RHODA</td>
<td>Carè et al, 2007</td>
<td>Modulates COX-2 and MPP-9 expression (Xue et al, 2008)</td>
</tr>
<tr>
<td>mir-133b</td>
<td>IGF1R</td>
<td>Targetsan/5.1/Ning et al, 2009</td>
<td>Member of IGF-1 pathway, beneficial effect on plaque stabilization (LeRoith et al, 1995)</td>
</tr>
<tr>
<td>mir-127</td>
<td>INSR (insulin receptor)</td>
<td>Targetsan</td>
<td>Member of IGF-1 pathway, beneficial effect on plaque stabilization (LeRoith et al, 1995)</td>
</tr>
<tr>
<td>mir-127</td>
<td>BCL6 (B-cell CLL/lymphoma 6)</td>
<td>Diana Lab/Saito et al, 2006</td>
<td>Modulation of vascular inflammation (Toney et al, 2000)</td>
</tr>
<tr>
<td>mir-127</td>
<td>NOX 1 (NADPH oxidase)</td>
<td>Targetsan</td>
<td>Modulation of vascular inflammation and oxidative stress (Lasségue et al, 2010)</td>
</tr>
<tr>
<td>mir-127</td>
<td>ADIPO (adiponectina)</td>
<td>Targetsan</td>
<td>Modulation of macrophage function and VSMCs proliferation (Otsuka et al, 2006)</td>
</tr>
<tr>
<td>mir-100</td>
<td>IGF1R (insulin-like growth factor 1 receptor)</td>
<td>Targetsan/5.1</td>
<td>Member of IGF-1 pathway, beneficial effect on plaque stabilization (Scheidegger et al, 2000)</td>
</tr>
<tr>
<td>mir-100</td>
<td>E2F1 (E2F transcription factor 1)</td>
<td>Targetsan/5.1</td>
<td>Modulation of vascular inflammation (Chen et al, 2002)</td>
</tr>
</tbody>
</table>

IGF-1 indicates insulin growth factor 1; VSMC, vascular smooth muscle cell; ECM, extracellular matrix; COX-2, cyclooxygenase 2; MPP-9, matrix metalloproteinase 9.

performed in a 96-well plate for 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and then held at 4°C. Subsequently, real-time PCR was conducted using a standard TaqMan PCR kit protocol on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems). The reactions were performed using a 1 μL reverse transcription product, 2× TaqMan Universal PCR Master Mix NO Amperase UNG, 0.2 μmol/L TaqMan Probe, 1.5 μmol/L forward primer, 0.7 μmol/L reverse primer, and incubated in a 96-well optical plate at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All reactions, including nitric oxide-template controls, were run in triplicate for each case for assessment of technical variability.

Cells Culture and Transfection
The protocol for umbilical cords harvesting was approved by the Ethics Committee of the Pescara Hospital in accordance with the principles of the Declaration of Helsinki. Umbilical cords were obtained from randomly selected healthy mothers delivering at the Pescara Hospital who had signed a written consent form. Primary human umbilical vein endothelial cells (HUVECs) were obtained and cultured as described previously. For transfection, HUVECs were grown to confluence in 6-well tissue culture plates. HUVECs were then incubated for 8 hours and 24 hours with 300 ng of hsa-mir-133a and hsa-mir-145 mimic separately. Control samples were incubated only with high performance reagent. The 2 timing points (8 hours and 24 hours) of the experiment have been chosen by considering both miRNA and protein half-life to better evaluate miRNA effect in this temporal slot.

Western Blot
For the Western blot analysis, HUVECs have been tripsynized, pelleted, and suddenly lysated with Ripa Buffer. Quantification of total lysate was obtained by Bradford method. Total cell lysates were

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Data Analysis
Relative quantification of miRNA expression was calculated with the 2-ΔΔCt method (Applied Biosystems, User Bulletin No. 2). Data were presented as log_{10} of relative quantity of target miRNA, normalized with respect to references as endogenous controls, and to a calibrator sample. The relative expression level of mature miRNAs was calculated according to geNorm by using 3 different miRNAs as references to determine the normalization factor and to correct differences in the amount of cDNA loaded into PCR reactions. The quantitative reverse transcription–PCR results were imported into an Excel file (Microsoft Corporation), and the average value of triplicate Ct values was converted to quantities for geNorm analysis. Results were thus represented as histograms reflecting the fold changes over the expression levels of the selected housekeeping genes.

Statistical Analysis
The mean levels of miRNA expression in asymptomatic and symptomatic plaques were compared using the independent-sample Student t test. A logistic regression analysis was performed including all variables in the statistical model to assess whether the molecular predictor variable (miRNA phenotype) could significantly predict the dichotomous clinical outcome (symptomatic/asymptomatic). The probability values were calculated with the likelihood-ratio χ^2 test. Probability values of <0.05 were considered significant. All statistical analysis was performed using the SPSS statistical package 15.0 for Windows.

Results
Pilot Study on MicroRNA Expression in Atherosclerotic Plaques
All the 41 miRNAs were expressed in all the plaques investigated. Normalization of data were obtained by the GeNorm method (see “Material and Methods”) using 3 of the 41 miRNA examined, mir125a, mir150, and mir134, that showed a constant low (mean 19.9), medium (mean 21.5), or high (mean 28.1) expression level, respectively, in the 15 plaques investigated. Expression data from the 5 miRNAs was logarithmic-transformed to stabilize the variance and correct the positively skewed distributions. After normalization, we found significant differences in the expression of 5 miRNAs (miRNA-100, miRNA-127, miRNA-145, miRNA-133a, miRNA-133b) in symptomatic versus asymptomatic plaques (Figure 1), whereas all the other miRNA analyzed were not significantly different between the 2 groups. All of these 5 miRNAs were found to be overexpressed in symptomatic plaques.

Expression of Selected miRNAs in an Independent Set of Atherosclerotic Plaques: Validation Study
After these results on the first subset of atherosclerotic plaques (Chieti cohort), we decided to validate the 5 poten-
Correlation Between miRNA Expression and Changes in Culprit Gene Expression

As described, we have selected the study miRNAs according to the involvement of their target genes in the pathophysiology of atherothrombosis (Table 2). Therefore, to demonstrate that differences in miRNA expression between symptomatic and asymptomatic plaques are really associated with significant perturbation in the expression of genes involved in plaque instability, we have performed additional quantitative reverse transcription–PCR experiments aimed to measure in the same plaques used for miRNA analysis the levels of mRNA for CD40, matrix metalloproteinase (MMP)-9 and MMP-13, relevant genes potentially controlled by culprit miRNAs (miRNA 145, 133a, and 127, respectively). Notably, curve estimation regression analysis showed a statistically significant inverse correlation between miRNA 145 and CD40 ($R=0.181$; $P=0.013$) and between miRNA 127 and MMP-13 ($R=0.174$; $P=0.014$) and a slightly nonsignificant correlation between miRNA 133a and MMP-9 gene expression ($R=0.104$; $P=0.063$).

Generation of a Predictive Model Based on the Expression of Multiple miRNA

A next step in our experimental approach was to integrate the data in an unique miRNA signature that could be usefully utilized as a predictive model in future applications. miRNA expression data were used as independent variables in a logistic regression model to predict the dichotomous clinical outcome (symptomatic/asymptomatic). When the 4 candidate variables (ie, miRNA-100, miRNA-127, miRNA-133a, and miRNA-133b; the 4 miRNAs resulted significantly more expressed in symptomatic plaques than asymptomatic ones) were entered, the overall model resulted statistically significant ($P<0.05$), and 36.4% of the total variability could be predicted by the selected independent variables. Considering a cutoff value of 0.5, the model was able to correctly predict 73.5% of cases (Table 3). When also data from miRNA-145 were entered in the model together with the other 4 variables, the regression model remained significant; 42.2% of the total variability could be predicted by the 5 selected independent variables; the model was able to correctly predict an overall 82.4% of cases (Table 3).

Confirmation of the Biological Relevance of Detected miRNAs: In Vitro Experiments on Transfected Cells

Finally, to confirm that study miRNAs may really influence at the protein level the expression of target genes, we performed additional in vitro studies on HUVECs incubated for 8 hours and 24 hours with hsa-mir-133a or hsa-mir-145 mimics. Notably, results confirm the ability of miRNAs in regulating target protein expression (Figure 3). Indeed, we found that after 8 hours incubation with miR-133a mimic, HUVECs show a downregulation of MMP-9 protein levels ($−49\%$ compared with control cells). Interestingly, this inhibition persisted also after 24 hours incubation ($−38\%$). Similarly, plasminogen activator inhibitor-1 protein levels were downregulated by miR-145. Indeed, after 8 hours and 24 hours incubation with miR-145 mimics, HUVECs expressed less plasminogen activator inhibitor-1 protein compared with control cells ($−38\%$ and $−63\%$, respectively). Noteworthy, we found that plasminogen activator inhibitor-1 may be downregulated also by miR-133a ($−44\%$ and $−58\%$ after 8 hours and 24 hours incubation, respectively) despite the fact that this aspect was not predicted by used target prediction algorithms. Finally, the specificity of our results was confirmed by the observation that PTGIS was unaffected by miR-133a, in agreement with the last update of miRNA target prediction algorithm reporting that PTGIS is no more considered a potential target gene for miR-133a (miranda, August 2010 release).

Discussion

It has been previously reported that modulation of an aberrantly overexpressed miRNA, miR-21, in arterial vessel can increase the risk of cardiovascular events by promoting neointimal lesion formation. In this report, we provide a further step forward by demonstrating that several miRNAs are constantly expressed in established atherosclerotic plaques in humans and that expression of one specific miRNA signature may contribute to plaque evolution toward an unstable phenotype.

To the best of our knowledge, this is the first study that extensively investigates the expression pattern of miRNAs in atherosclerotic plaques in humans. In particular, we demonstrated for the first time that: (1) miRNAs are expressed in human atherosclerotic plaques; (2) one specific miRNA signature is constantly associated with an unstable plaque phenotype leading to plaque rupture and acute clinical events such as stroke; and (3) conventional risk factors per se seem to be not responsible for the induction of this specific miRNA signature in human atherosclerotic plaques.

In this study, we present evidence for the diffuse expression of several miRNAs in advanced atherosclerotic plaques in humans. Before our work, no studies had investigated the expression of miRNAs in human atherosclerotic plaques.

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Table 3. Predictive Model of Clinical Outcome Based on the Expression of Multiple miRNAs

<table>
<thead>
<tr>
<th>Observed</th>
<th>Predicted by 4 miRNAs</th>
<th>Percentage Correct</th>
<th>Predicted by 5 miRNAs</th>
<th>Percentage Correct</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asymptomatic</td>
<td>Symptomatic</td>
<td></td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>17</td>
<td>4</td>
<td>81.0</td>
<td>19</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>5</td>
<td>8</td>
<td>61.5</td>
<td>4</td>
</tr>
<tr>
<td>Overall</td>
<td>73.5</td>
<td></td>
<td></td>
<td>82.4</td>
</tr>
</tbody>
</table>

miRNAs indicates microRNAs.
whereas only one study\textsuperscript{11} had examined the expression of miRNAs in dysfunctional vessels in rats. In this elegant miRNA profiling study, Ji et al found that expression of the antiapoptotic miRNA miRNA-21\textsuperscript{27,28} increased rapidly after carotid balloon injury in rats, suggesting a role for miRNA-21 in neointimal growth after arterial injury. Now we provide clear evidence that miRNAs are widely expressed not only in artificially and acutely injured vessels, but also in advanced atherosclerotic plaques, and not only in rats, but also in humans.

One of the most important finding of this study is that 4 critical miRNAs were significantly more expressed in atherosclerotic plaques from patients with recent acute ischemic stroke (a clinical model of vulnerable plaque) with respect to plaques obtained from clinically asymptomatic subjects (a clinical model of stable plaque). In fact, we found that $>71\%$ of patients affected by stroke had miRNA expression levels higher than the mean expression levels of corresponding miRNAs in asymptomatic patients. This information may be relevant for at least two reasons. The first is that, since we well know that carotid plaques from patients with acute stroke represent the prototype of complex lesions highly prone to rupture, so the observation of a higher prevalence of these 4 miRNAs in this kind of plaque relates the presence of these miRNAs in human plaque to an unstable plaque phenotype and to an increased risk of future acute ischemic events precipitated by plaque rupture. The second is that, since the discovery of new therapies for plaque stabilization still represents the main mission of cardiovascular research, with this study, by identifying the specific miRNA signature for stroke, now we could be able to use computer algorithms to identify previously unrecognized molecular targets. This approach could ultimately lead to innovative therapeutic approaches in stroke.

The observation that an unique miRNA signature is constantly expressed in vulnerable plaques raises the question if this phenomenon is the consequence of a local factor, i.e., a particular inflammatory milieu present in some plaques perhaps regulated by specific genetic traits, or in contrast, it is the consequence of systemic and more generalized factors. In this light, the demonstration that the constant expression of this specific miRNA signature in vulnerable plaques was not associated with a higher presence of any specific risk factor (diabetes, hypertension, dyslipidemia, smoking) clearly supports the view that local factors or genetic traits per se rather than metabolic factors are responsible for miRNA regulation in cells infiltrating vulnerable plaques.

In this study, we found that the expression patterns of miRNA-100, miRNA-127, miRNA-145, miRNA-133a, and miRNA-133b were always comparable to each other; therefore, plaques that were highly positive in 1 of the 4 miRNAs were also highly positive for the remaining 3 miRNAs, and no plaque was found to be positive for only 1 of these miRNAs. We believe this observation is of interest, because it seems to suggest that miRNA-100, miRNA-127, miRNA-133a, and miRNA-133b are concomitantly expressed in the same cells in the plaques and that complex regulatory mechanism(s) intimately linking these 4 miRNAs may exist.

Recently, the growing number of genes potentially implicated in plaque homeostasis and identified as regulated (repressed or also activated\textsuperscript{29}) by miRNAs\textsuperscript{30,31} has led to the supposition that miRNAs can have a deep impact on athero-

![Western Blot analysis showing the expression of target protein in HUVECs transfected with miR mimics. Results are expressed as percentage reduction compared with control cells. HUVECs indicates human umbilical vein endothelial cells.](image-url)
sclerotic plaque development and instability. In this light, it is quite remarkable that the 4 miRNAs found to be part of the unique miRNA signature, which characterize the clinically symptomatic plaques, may regulate several critical aspects of plaque evolution, as reflected by the bioinformatic analysis depicted in Table 2. In particular, the genes regulated by these 4 miRNAs are deeply involved in the modulation of inflammation (ie, CD40, BCL6), apoptosis (ie, caspase-9, E2F1), oxidative stress (ie, NOX1), vascular smooth muscle cells dedifferentiation and proliferation (KLF-15 and platelet-derived growth factors), extracellular collagen turnover (MMP-9), eicosanoid metabolism (PTGIS, CDC42, and RhoA), thrombosis (ie, plasminogen activator inhibitor-1), and reverse cholesterol transport (ie, ABCA1).

Whereas this is the first in vivo evidence suggesting a role for miRNA-100 and miRNA-127 in cardiovascular diseases, on the contrary, several previous in vitro and in animal studies have emphasized the involvement of miRNA-145 and miRNA-133 in cardiovascular diseases. In fact, when miR-145 was experimentally overexpressed in vascular smooth muscle cells of the carotid artery after balloon injury, it decreased the rate of neointima formation.32 In addition, Xu et al33 and Latronico et al34 have recently reported an influence of miRNA-133 on cardiac muscle hypertrophy: reduced miRNA-133 expression was found in the ventricular tissue of mice subjected to transverse aortic constriction and in cultured cardiomyocytes treated with hypertrophic stimuli as well as in myocytes originating from hearts of cardiomyopathic patients. miRNA-133 gene targets have been also studied. Between the numerous possible targets, the reported influence of miRNA-133 (as well as miRNA-127 and miRNA-145) on the expression of MMP-9, MMP-13, and MMP-15 is critical in the setting of plaque destabilization. In fact, whereas expression of MMPs in endothelial cells is beneficial as part of the endogenous fibrinolytic system, in contrast, MMP (particularly MMP-9) activity within atherosclerotic plaque causes collagen digestion leading to plaque cap weakness and ultimately to plaque rupture. Furthermore, expression of platelet-derived growth factor D, CDC42, and Rho-A (both GTP-GDP-binding molecules) may be also relevant in the vascular scenario; because all these genes are deeply involved in cell growth32,36,37. Again, a strong antiapoptotic function is attributed to miRNA-133, because it has caspase-9 as a validated target.29 Finally, a report from Tuccoli and colleagues focused on the direct effect of miRNA-133 not only on muscle cells, but also on endothelial cells.38 In this study, an analysis of miRNA expression in HUVECs revealed that miRNA-133, when overexpressed, has angiogenic factor receptors as putative targets, thus demonstrating a potential role of this miRNA even in the control of the vascular angiogenesis.

Lastly, in our study, the in vitro demonstration that identified miRNA signature may really influence the expression of target genes at protein level support the clinical relevance of our observation and rules out the hypothesis that miRNA alteration in symptomatic plaques is simply an epiphenomenon without any practical readout. Some aspects may represent strength points of the study. In particular, we believe that the observation that one specific miRNA signature characterizes plaques prone to rupture in two different groups of patients recruited in two different hospitals by two totally independent surgical teams is very important, because it rules out the possibility that results were influenced by variables such as diagnostic procedures, specific surgical techniques, and concomitant medical therapies. On the other hand, the constant identification in all the symptomatic plaques of a specific complex miRNA signature linking 4 different miRNAs rather than the occasional discovery of a single miRNA supports the scientific quality of this observation and argues against any influence of chance in our observation.

Nevertheless, some limitations are present in this study. The most important is that, based on our data, we can attribute the discovered miRNA signature to an active role in the process of plaque destabilization only by indirect evidence (ie, higher expression of this miRNA signature in clinically symptomatic plaques), but cannot exclude that the changes observed in miRNA are a consequence rather than a cause of stroke. However, the definitive role of these miRNAs in plaque instability will be definitively confirmed only by future interventional in vivo studies involving selective miRNA modulators. Furthermore, in this study, we did not provide information regarding the specific area of the plaque expressing these critical miRNAs as well as the specific cellular source(s). Finally, we did not provide any information regarding the molecular mechanism(s) involved in the regulation of this peculiar miRNA signature.

Despite the described limitations we believe that our findings are potentially important from a fundamental standpoint because they indicate a potential role for miRNAs in the homeostasis of atherosclerotic plaques in humans. From a practical standpoint, these findings identify a new potential pharmacological target for plaque stabilization and raise the interesting possibility, which needs to be confirmed by future intervention studies with antisense oligonucleotides, that selective modification in this miRNA signature might provide a novel form of therapy for plaque stabilization in humans.

Acknowledgments
We thank Dr Sara Di Silvestre and Francesca Pietrucci for technical support, Dr Daniele Pavone for statistical assistance, and Dr Luciano Carbonari for clinical support.

Sources of Funding
This study was supported by academic research funds.

Disclosures
None.

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*Stroke.* published online August 4, 2011;
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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