Carotid Plaque Rupture Is Accompanied by an Increase in the Ratio of Serum circR-284 to miR-221 Levels

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Background—Atherosclerotic plaque rupture is accompanied by an acute decrease in the carotid plaque expression of micro-RNAs (miRs)-221 and miR-222. Circular RNA (circR)-284 is a potential inhibitor of miR-221/miR-222 activity. We aimed to determine whether changes in the serum levels of these noncoding RNAs are observed in patients with asymptomatic high-grade carotid disease versus patients with acutely symptomatic carotid disease and recent ischemic stroke. Additionally, we tested the use of functionally related noncoding RNA pairs to enhance the discriminatory power of noncoding RNAs as circulating biomarkers.

Methods and Results—Serum levels of miR-221, miR-222, miR-145, and circR-284 were measured in 24 asymptomatic (asymptomatic) and 17 acutely symptomatic patients ([urgent] ischemic cerebrovascular event within the previous 5 days) undergoing carotid endarterectomy. miR-221 was significantly lower, whereas circR-284 was elevated in the serum of the urgent compared with the asymptomatic group. The ratio of serum circR-284:miR-221 was significantly elevated in the urgent group (P=0.0002) and exhibited favorable characteristics as a biomarker indicative of carotid plaque rupture and stroke. A validation study in 112 patients (47 asymptomatic, 41 urgent, and 24 patients with a cerebrovascular event between 5 and 180 days of the carotid endarterectomy [symptomatic]) confirmed elevation of serum circR-284:miR-221 uniquely in the urgent group (P<0.001) and favorable sensitivity and specificity for detecting plaque rupture and stroke.

Conclusions—Serum circR-284:miR-221 has potential as a diagnostic biomarker of carotid plaque rupture and stroke. Moreover, we demonstrate the use of functionally related pairs of circulating noncoding RNAs as biomarkers in cardiovascular disease. **(*Circ Cardiovasc Genet*. 2017;10:e001720. DOI: 10.1161/CIRCGENETICS.117.001720.)**

Key Words: endarterectomy, carotid ■ ischemia ■ microRNAs ■ RNA, untranslated ■ stroke

Early detection of acute ischemic stroke has the potential to reduce morbidity and mortality in patients with advanced carotid atherosclerotic disease.[1] Currently, there is no biomarker predictive of atherosclerotic plaque rupture and stroke. Stable carotid atherosclerotic plaques are characterized by a necrotic core with an overlying fibrous cap composed of VSMCs (vascular smooth muscle cells) in a collagen-rich matrix.[2] The fibrous cap results from intimal thickening in response to arterial inflammation.[3] In vulnerable plaques, the fibrous cap is thinner, exhibiting fewer VSMCs and increased inflammatory cells. Therefore, a promising strategy for developing diagnostics predictive of a future carotid-related ischemic cerebrovascular event (eg, transient ischemic attack and ischemic stroke) is identifying circulating biomarkers indicative of a transition from intimal thickening to fibrous cap thinning.

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Noncoding RNAs (ncRNAs) have emerged as important effectors of intimal thickening and potential circulating biomarkers for cardiovascular disease.[4–6] Recently, we reported that miR-221 and miR-222, but not miR-145, are reduced in the shoulder region of carotid plaques after an acute ischemic cerebrovascular event.[7] miR-221 and -222 promote intimal thickening through downregulation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor that inhibits VSMC cell cycle progression.[8,9] miR-145 inhibits intimal thickening through promotion of VSMC differentiation.[10] A second form of ncRNA, circular RNAs (circR), are formed when the 5′ and 3′ ends of a single RNA strand are spliced.[11] Recently, Memczak et al[12] identified a large number of circRNAs including circR-284 (circbase.org), which possesses an miR-221 and miR-222 binding site and may serve to regulate miR-221/miR-222 activity.

Here, we report that serum miR-221 levels are reduced after plaque rupture, which parallels our recent findings in the carotid plaque.[7] Furthermore, we found that circR-284 is expressed in the carotid plaque and is increased in the sera of patients after plaque rupture. Our data demonstrate that the serum ratio of circulating circR-284 to miR-221 is elevated in patients presenting with an acute carotid-related ischemic

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event and has the potential to serve as a diagnostic biomarker for carotid-related cerebrovascular ischemia.

Materials and Methods

Patient Population

Discovery (n=41) and validation (n=112) studies were conducted with serum collected from patients undergoing carotid endarterectomy (CEA) in the Section of Vascular/Endovascular Surgery, Department of Surgery at the Ochsner Clinic. Patients were stratified into 3 groups: (1) those without a previous cerebrovascular event in the previous 6 months (asymptomatic), (2) patients with either an acute stroke or transient ischemic attack within 5 days of the CEA (urgent), and (3) patients who had a more remote cerebrovascular ischemic event within 5 to 180 days of the CEA (symptomatic). The Ochsner Institutional Review Board approved the protocol and informed consent was obtained from all patients.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from the serum using the miRNeasy Serum/Plasma kit (Qiagen Inc., Valencia, CA) with minor modifications. miRNAs were measured using the miScript II RT Kit coupled with the miScript SYBR Green PCR Kit (Qiagen). The catalog numbers for the individual miRNA PCR assays are listed in the Table I in the Data Supplement. Convergent and divergent primers published previously by Memczak et al[12] were used to confirm expression and compare serum levels of circR-284. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The ratio of circR-284:miR-221 was calculated as $2^{-(\Delta\Delta Ct)}$ with $\Delta C_t = C_{t\,(miR\text{-}221)} - C_{t\,(circR\text{-}284)}$ and $\Delta\Delta C_t = \Delta C_{t\,(urgent)} - \Delta C_{t\,(asymptomatic)}$.

Droplet Digital PCR

The QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories) was used in combination with the primers for miR-221 and circR-284. For measurement of miR-221, the cDNA was prepared in the same manner as above and the entire reaction was used as template, according to the manufacturer's instructions. For measurement of circR-284, a 1-step PCR protocol was used where a reverse transcription step (50°C, 30 minutes) was inserted before the manufacturer's recommended PCR protocol. The PCR was performed on a C1000 Touch Thermal Cycler and the QX200 Droplet Digital PCR system (Bio-Rad Laboratories).

Statistics

Data are expressed as the mean±SEM. Statistical analysis between groups was performed using Student *t* test or ANOVA coupled with Tukey honest significant difference test. χ^2 analysis was used to compare categorical variables across groups. With 112 patients in the validation study, it was appropriately powered to achieve α=0.05 and β=0.019. Accuracy was calculated as the number of true positives plus false-negatives divided by the total number of samples. All analyses were performed using SPSS version 19.0 (IBM).

Results

Discovery Study Population

Serum levels of miR-145, miR-221, miR-222, and circR-284 were measured in 24 asymptomatic patients and 17 urgent patients while undergoing CEA. In the urgent group, the average time between the cerebrovascular event and CEA was short (2.6±0.3 days) and the average stroke severity was 3.1±1.0 on the National Institutes of Health Stroke Scale, representing minor strokes. There were no significant differences in age, sex, smoking status, body mass index (BMI), lipid panel, use of antiplatelet or anticoagulant therapies, or serum creatinine between the asymptomatic and urgent CEA groups (Table 1).

Table 1. Characteristics of Patients in the Discovery Study

Characteristics	Total (n=41)	Asymptomatic (n=24)	Urgent (n=17)	*P* Value
Age, y	68.4±1.9	69.1±2.4	67.5±3.2	0.68
Male sex	30 (73)	16 (67)	14 (82)	0.20
Body mass index, lb/in2	27.1±0.9	27.0±1.3	27.1±1.2	0.99
Total cholesterol, mg/dL	158.7±9.0	152.9±14.1	162.8±12.0	0.60
HDL, mg/dL	44.4±2.0	45.3±3.8	43.7±2.2	0.70
LDL, mg/dL	93.6±5.3	86.2±10.0	98.9±5.5	0.28
Triglycerides, mg/dL	118.3±11.0	107.1±14.8	126.2±15.7	0.38
Serum creatinine, mg/dL	1.1±0.1	1.0±0.1	1.1±0.1	0.52
Smoker	9 (22)	5 (21)	4 (24)	0.71
Anticoagulant use	27 (65)	19 (79)	9 (47)	0.05
Antiplatelet use	15 (37)	11 (41)	4 (24)	0.20
Time to CEA, d	…	…	2.6±0.3	…

CEA indicates carotid endarterectomy; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

Serum miR-221

Recently, we reported that miR-221 and miR-222 are reduced in the carotid plaque shoulder region of patients immediately after an acute plaque rupture and ischemic cerebrovascular event.[7] To determine whether these changes are also reflected in the serum, we compared circulating levels of miR-221, miR-222, and miR-145 in the asymptomatic and urgent groups. MiR-222 and miR-145 were not reliability detected in the majority of our samples. The urgent group exhibited a significantly lower level of miR-221 than the asymptomatic group (0.25±0.11 versus 1.00±0.31; *P*=0.01; Figure 1A).

Serum circR-284

As circR-284 has a potential miR-221 binding site (Figure I in the Data Supplement), it is a potential inhibitor of miR-221 and miR-222, making it a candidate for normalization of miR-221 measurements. We confirmed that circR-284 is expressed in human VSMCs (Figure II in the Data Supplement) and in carotid plaques (data not shown). Serum circR-284 levels did not exhibit a significant change after an acute carotid plaque rupture-mediated ischemic cerebrovascular event, such as transient ischemic attack or stroke (2.96±1.16 versus 1.00±0.37; *P*=0.06; Figure 1B).

Serum circR-284:miR-221

Receiver operator characteristic curve analysis suggested miR-221 levels do not sufficiently discriminate between the asymptomatic and urgent groups to serve as a predictor of plaque rupture. Given the inverse putative functional relationship between miR-221 and circR-284, we tested whether the ratio of circR-284 to miR-221 (circR-284:miR-221) would yield a serum biomarker of a carotid-related cerebrovascular ischemic event. Serum circR-284:miR-221 was significantly higher in the urgent than in the asymptomatic group (11.7±0.48 versus 1.0±0.6; *P*=0.0002; Figure 1C). There were

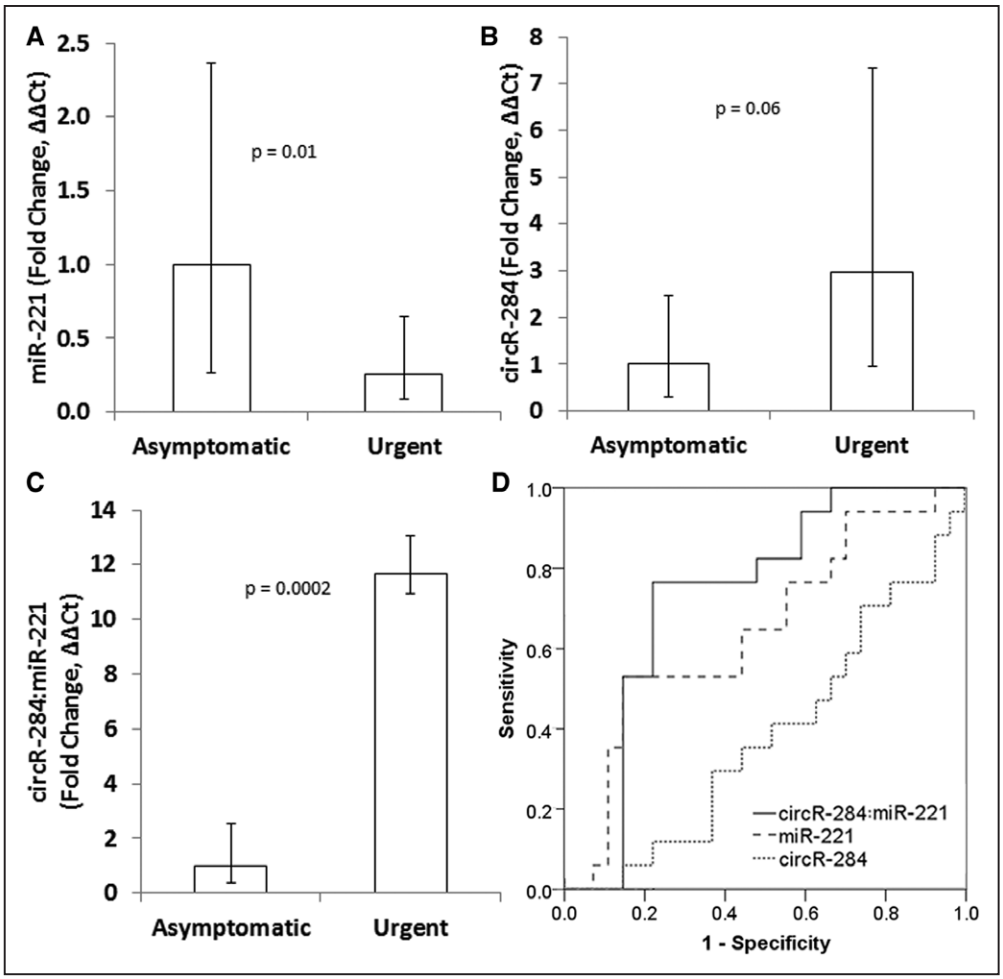

Figure 1. Serum levels of miR-221, circR-284, and circR-284:miR-221 ratio in the discovery study (n=41 total). **A–C**, Quantification of miR-221, circR-284, and circR-284:miR-221 ratio in the serum of asymptomatic (n=24) and urgent (n=17) patients. **D**, Receiver operator characteristic curve analysis of miR-221, circR-284, and circR-284:miR-221 ratio as an indicator of a recent ischemic cerebrovascular event.

not significant differences in serum circR-284:miR-221 with respect to sex, history of smoking, statin use, race, use of antiplatelet or anticoagulant therapies, and presence of chronic kidney disease nor was there a significant correlation between the serum ncRNAs levels and age, BMI, lipid panel, or serum creatinine (Tables II and III in the Data Supplement). Comparison of the sensitivity, specificity, likelihood ratios, accuracy, and receiver operator characteristic curves of the miR-221 and circR-284:miR-221 tests demonstrates that circ-284:miR-221 has potential as a diagnostic test for carotid plaque rupture leading to an acute ischemic event (Figure 1D; Table 2).

Validation Study

Next, we sought to validate the use of the ratio of circulating circR-284:miR-221 as a biomarker for the occurrence of an acute ischemic cerebrovascular event after carotid plaque rupture. To do this, we measured circulating miR-221 and circR-284 in an additional set of 48 asymptomatic, 41 urgent, and 24 symptomatic patients using droplet digital PCR. There was no significant difference between the groups for age, BMI, lipids, serum creatinine, use of antiplatelet or anticoagulant therapies, or smoking history and only a significantly greater percentage of males in the urgent group (Table 3). Compared with both the asymptomatic and symptomatic groups, the urgent group exhibited a trend toward lower serum miR-221 (9.1±0.67 versus 13.2±1.52 and 11.0±2.27 copies/ng of RNA; P=0.10 compared with the asymptomatic and P=0.63 compared with symptomatic; Figure 2A) and significantly elevated serum circR-284 (31.6±2.09 versus 9.3±1.40 and 10.0±2.89 copies/ng of RNA; P<0.001 for both comparisons; Figure 2B). Thus, although miR-221 expression is greater than circR-284 expression in the asymptomatic and symptomatic groups, the opposite is true in the urgent group. As expected, these values are orders of magnitude lower than those seen with tissue or cultured VSMCs. Circulating circR-284:miR-221 was highest in the urgents (4.2±0.40 versus 0.6±0.05 and 0.7±0.1, P<0.001 for urgent versus asymptomatic and symptomatic groups; Figure 2C). Neither were there no significant differences in serum circR-284:miR-221 with respect to sex, history of smoking, statin use, use of antiplatelet or anticoagulant therapies nor was there a significant correlation between the serum ncRNAs levels and BMI, lipid panel, or serum creatinine (Tables IV and V in the Data Supplement). There was a mild but significant correlation between serum circR-284:miR-221 ratio and age (R^2 = 0.04; P=0.05). Multivariable logistic regression confirmed a significant association between a recent carotid

Table 2. Evaluation of Biomarkers in Discovery Study

Marker	AUC			Sensitivity	Specificity	LR +	LR–	Accuracy, %
	Value	95% CI	*P* Value					
miR-221	0.72	0.55–0.89	0.017	0.59	0.63	1.57	0.66	61
circR-284	0.65	0.49–0.82	0.095	0.53	0.67	1.59	0.71	61
circR-284:miR-221	0.82	0.69–0.96	<0.001	0.76	0.88	6.12	0.27	83

AUC indicates area under the curve; CI, confidence interval; LR+ likelihood ratio positive; and LR–, likelihood ratio negative.

plaque rupture and an increased serum circR-284:miR-221 ratio (*P*=0.01; Table VI in the Data Supplement) after adjustment for age, sex, serum lipids, serum creatinine, BMI, hypertension, and smoking status. As in the discovery study, comparison of the sensitivity, specificity, likelihood ratios, accuracy, and receiver operator characteristic curves of the miR-221, circR-284, and circR-284:miR-221 levels demonstrate that the circ-284:miR-221 ratio has a greater potential as a diagnostic test for an acute ischemic cerebrovascular event after carotid plaque rupture (Figure 2D; Table 4).

Discussion

The goal of this study was to determine whether circulating ncRNAs could be used to identify those patients who have experienced a recent carotid-related cerebrovascular ischemic event, allowing for minimization of the time between the event and accurate diagnosis, leading to earlier treatment. Recently, we reported that miR-221 and miR-222 expression in the carotid plaque shoulder region is reduced immediately after a carotid-related ischemic cerebrovascular event.[7] Here, we report that serum levels of miR-221 are decreased after an ischemic cerebrovascular event, comparable to our previous observation in the carotid plaque itself. To overcome the need for normalization of serum ncRNAs, we paired miR-221 with a putatively related ncRNA, circR-284, to achieve a serum biomarker with the potential to address this clinical need.

This report, although focused on the use of these ncRNAs as serum biomarkers of plaque rupture, raises additional questions about the molecular mechanism regulating their expression and secretion to the serum. Our data combined with others confirm that both miR-221 and circR-284 are expressed in VSMCs.[9,13] Located in different regions of the genome, the expression of these RNAs is likely independent of each other. The proposed relationship between miR-221 and circR-284 is instead based on the presence of a miR-221 binding site in the circR-284 sequence. The presence of this sequence suggests that circR-284 may act as a miR-221 sponge, inhibiting its activity. In addition, the mechanism regulating serum levels of these ncRNAs remains unclear. As a first step in determining whether the carotid plaque itself is the source of the circulating ncRNAs, we have measured both miR-221 and circR-284 in microparticles isolated from cultured VSMCs (Figure III in the Data Supplement). We are actively examining these issues with ongoing studies in our laboratory.

One characteristic of miR-221 that makes it an attractive candidate for use as a serum biomarker for carotid plaque rupture is that expression in the carotid plaque returns to normal levels within 7 days post-event.[7] Data from the validation study, which included 24 patients with a cerebrovascular event within 5 to 180 days of the CEA (symptomatic), in addition to the 47 asymptomatic and 41 urgent patients, demonstrate that serum miR-221 and circR-284 exhibit a similar temporal pattern as the carotid tissue. These ncRNAs show a trend toward increasing miR-221 and decreasing circR-284 levels in the symptomatic group. Previously, Tsai et al[14] reported a similar, but smaller, decrease in serum miR-221 in patients after a stroke or transient ischemic attack within the previous 7 days. The difference observed is likely because of the shorter time interval between the ischemic cerebrovascular event and sample collection in our study (2.6 days in our study versus ≈7 days for the study of Tsai et al[14]). This transient change in expression yields the ability of serum circR-284:mir-221 to discriminate between both asymptomatic and symptomatic

Table 3. Characteristics of Patients in the Validation Study

Characteristics	Total (n=112)	Asymptomatic (n=47)	Urgent (n=41)	Symptomatic (n=24)	*P* Value
Age, y	69.2±6.7	67.9±1.6	70.4±1.5	69.5±2.3	0.14
Male sex	75 (67)	25 (53)	33 (71)	17 (47)	0.02
Body mass index, lb/in^2	28.0±2.7	27.6±0.9	28.2±1.1	28.6±1.1	0.61
Total cholesterol, mg/dL	172.0±18.3	167.9±6.3	174.3±9.7	175.1±13.7	0.67
HDL, mg/dL	44.2±4.6	46.2±2.3	42.5±2.3	43.9±3.3	0.26
LDL, mg/dL	99.7±10.6	92.8±2.3	105.3±7.2	100.9±11.2	0.48
Triglycerides, mg/dL	141.0±14.7	140.2±18.6	149.5±13.2	122.5±10.2	0.51
Serum creatinine, mg/dL	1.1±0.1	1.0±0.0	1.1±0.1	1.0±0.1	0.33
Smoker	75 (67)	32 (68)	28 (68)	15 (63)	0.98
Anticoagulant use	90 (80)	42 (89)	29 (71)	19 (79)	0.09
Antiplatelet use	41 (37)	20 (43)	12 (29)	9 (38)	0.43

HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein

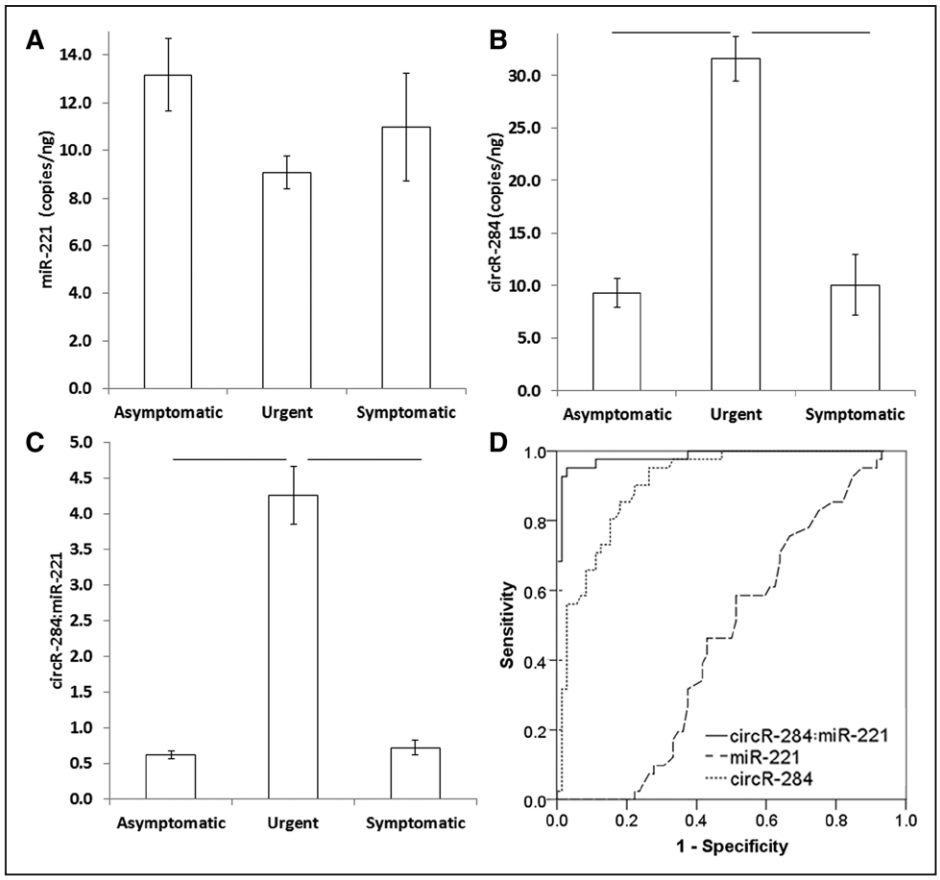

Figure 2. Serum levels of miR-221, circR-284, and circR-284:miR-221 ratio in the validation study (n=112 total). **A–C**, Quantification of miR-221, circR-284, and circR-284:miR-221 ratio in the serum of asymptomatic (n=47), urgent (n=41), and symptomatic (n=24) patients. **D**, Receiver operator characteristic curve analysis of miR-221, circR-284, and circR-284:miR-221 ratio as an indicator of a recent ischemic cerebrovascular event. Bar indicates $P<0.05$.

patients and permits identification of patients with a recent (within 5 days) cerebrovascular event, urgents in our study.

Normalization of serum measurements of individual ncRNAs is difficult as the standard housekeeping genes are not reliable in serum.[15,16] Spike-in controls, such as *Caenorhabditis elegans* miR-39, are commonly used to control for differences in purification efficiencies. This method does not adjust for differences in blood volume. We examined the use of a spike-in control in the discovery study, but this did not enhance our data (data not shown). We then tested whether a potential functionally related ncRNA could, in combination with miR-221, serve as a biomarker of carotid plaque rupture. Although serum circR-284 alone does not seem promising as a biomarker, we demonstrate that the ratio of circR-284 to miR-221 is significantly increased after plaque rupture and exhibits sensitivity and specificity that suggest the ability to discriminate between urgent and asymptomatic patients. Beyond this clinical setting, pairs of functionally related ncRNAs as circulating biomarkers may yield more accurate biomarkers than standard normalization methods.

Minimizing the time between plaque rupture and treatment is critical in reducing the morbidity and mortality in this patient population. Patients diagnosed within 3 hours may be offered systemic thrombolysis with recombinant tissue-type plasminogen activator, which has improved outcomes after stroke.[1] However, tissue-type plasminogen activator utilization rates among patients with acute ischemic stroke is currently only 7% because of delay in diagnosis of >3 hours.[17] New methodologies aimed at creating a point-of-care device for measuring nucleic acids rapidly are being developed.[18–20] Pairing of such methodologies with our findings could create a rapid diagnostic that would have the potential to minimize diagnostic delays, aid in patient selection for possible thrombolysis, and, henceforth, increase tissue-type plasminogen activator utilization rates, improving outcomes.

Table 4. Evaluation of Biomarkers in the Validation Study

	AUC							
Marker	Value	95% CI	*P* Value	Sensitivity	Specificity	LR +	LR–	Accuracy, %
miR-221	0.47	0.36–0.57	0.564	0.49	0.51	0.99	1.01	50
circR-284	0.91	0.86–0.96	<0.001	0.80	0.85	5.34	0.23	83
circR-284:miR-221	0.98	0.96–1.00	<0.001	0.93	0.97	33.83	0.08	96

AUC indicates area under the curve; CI, confidence interval; LR + likelihood ratio positive; and LR– likelihood ratio negative.

Summary

The current data support the development of serum circR-284:miR-221 as a diagnostic biomarker for carotid-related ischemic stroke. There is a possibility for serum circR-284:miR-221 to serve as a diagnostic biomarker for atherosclerotic plaque rupture in additional arterial beds or as a prognostic biomarker of stroke risk, which could be assessed with future longitudinal studies. In addition, the functional relationship of circR-284 and miR-221 remains to be elucidated and is a current focus in our laboratory. We also show that the use of functionally related pairs of ncRNAs may serve as a powerful method for overcoming the issues with normalization of serum RNA levels. Overall, the data provide a basis for further studies testing the use of circulating levels of circR-284 and miR-221 as a diagnostic biomarker for ischemic cerebrovascular events.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Atherosclerotic plaque rupture in the carotid leads to stroke and in the coronary to a myocardial infarction. Animal models of atherosclerosis mimic the mature plaque (American Heart Association type V), but there are no reliable animal models for plaque rupture (American Heart Association type VI), hampering the identification of the molecular events associated with rupture of the thin fibrous cap. To address this, we use a translational model based on carotid plaque and sera from patients with asymptomatic, high-grade carotid stenosis (>80%) and those presenting with acute neurological symptoms (stroke) undergoing urgent carotid endarterectomy. We previously showed noncoding RNA changes occurring in recently ruptured plaques, a loss of microRNA-221 (miR-221), and we now demonstrate that these changes also occur in the sera. Moreover, circular RNA-284 (circR-284), which contains seed sequences that may act as a 'sponge' to regulate miR-221, is increased in the sera of acutely symptomatic carotid patients. Using the ratio of circR-284:miR-221, we are able to discriminate acutely ruptured from asymptomatic carotid patients. Such a serum biomarker may help to identify and treat patients who present with an acutely ruptured plaque and stroke in an expedited fashion and offer them time-sensitive therapy, such as systemic thrombolysis within the 3- to 4.5-hour time window. Future research will address whether these serum changes are associated with thinning of fibrous cap to a preruptured event. If so, it could help identify the patient with asymptomatic, high-grade carotid stenosis who is high risk and offer such patient more intensive medical therapy or a prophylactic carotid revascularization.

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Carotid Plaque Rupture Is Accompanied by an Increase in the Ratio of Serum circR-284 to miR-221 Levels

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SUPPLEMENTAL MATERIAL

Supplementary Figures

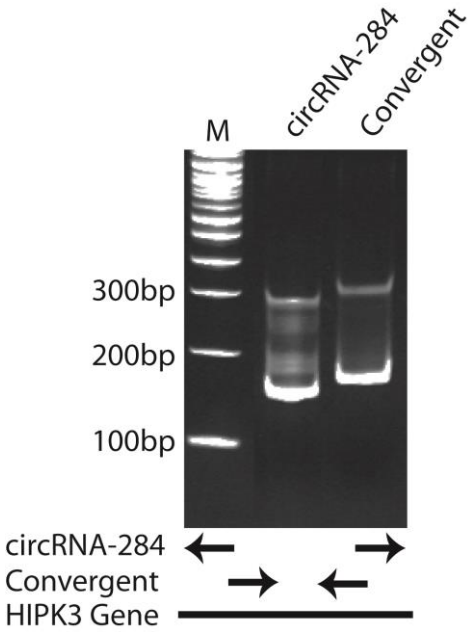
A.

1 GGTATGGCCT CACAAGTCTT GGTCTACCCA CCATATGTTT ATCAAACTCA GTCAAGTGCC
61 TTTTGTAGTG TGAAGAAACT CAAAGTAGAG CCAAGCAGTT GTGTATTCCA GGAAAGAAAC
121 TATCCACGGA CCTATGTGAA TGGTAGAAAC TTTGGAAATT CTCATCCTCC CACTAAGGGT
181 AGTGCTTTTC AGACAAAGAT ACCATTTAAT AGACCTCGAG GACACAACTT TTCATTGCAG
241 ACAAGTGCTG TTGTTTTGAA AAACACTGCA GGTGCTACAA AGGTCATAGC AGCTCAGGCA
301 CAGCAAGCTC ACGTGCAGGC ACCTCAGATT GGGGCGTGGC GAAACAGATT GCATTTCCTA
361 GAAGGCCCCC AGCGATGTGG ATTGAAGCGC AAGAGTGAGG AGTTGGATAA TCATAGCAGC
421 GCAATGCAGA TTGTCGATGA ATTGTCCATA CTTCCTGCAA TGTTGCAAAC CAACATGGGA
481 AATCCAGTGA CAGTTGTGAC AGCTACCACA GGATCAAAAC AGAATTGTAC CACTGGAGAA
541 GGTGACTATC AGTTAGTACA GCATGAAGTC TTATGCTCCA TGAAAAATAC TTACGAAGTC
601 CTTGATTTTC TTGGTCGAGG CACGTTTGGC CAGGTAGTTA AATGCTGGAA AAGAGGGACA
661 AATGAAATTG TAGCAATCAA AATTTTGAAG AATCATCCTT CTTATGCCCG TCAAGGTCAA
721 ATAGAAGTGA GCATATTAGC AAGGCTCAGT ACTGAAAATG CTGATGAATA TAACTTTGTA
781 CGAGCTTATG AATGCTTTCA GCACCGTAAC CATACTTGTT TAGTCTTTGA GATGCTGGAA
841 CAAAACTTGT ATGACTTTCT GAAACAAAAT AAATTTAGTC CCCTGCCACT AAAAGTGATT
901 CGGCCCATTC TTCAACAAGT GGCCACTGCA CTGAAAAAAT TGAAAAGTCT TGGTTTAATT
961 CATGCTGATC TCAAGCCAGA GAATATTATG TTGGTGGATC CTGTTCGGCA GCCTTACAGG
1021 GTTAAAGTAA TAGACTTTGG GTCGGCCAGT CATGTATCAA AGACTGTTTG TTCAACATAT
1081 CTACAATCTC GGTACTACAG

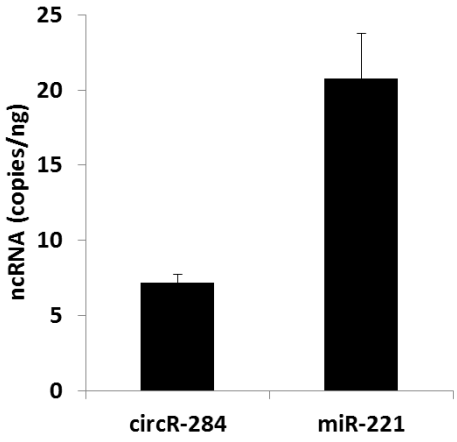
B.

```
circR-284 (position 356)    5'  U    AGAA     CCCCA                G 3'
                                CCU      GGC      GCGAUGUGGAUU
                                GGA      CCG      UGUUACAUCUAA
miR-221                     3' U             UA                   A 5'
```

Supplementary Figure I. circR-284 contains a binding site for miR-221. A) Sequence of circR-284 (A) with the miR-221 binding site highlighted in yellow. B) Binding of miR-221 and circR-284 as determined by RNAHybrid.[1]



Supplementary Figure II. Expression of circR-284 in human vascular smooth muscle cells. Two pairs of primers reported by Memczak et al.[2] were used to demonstrate the presence of circR-284 in total RNA isolated from human vascular smooth muscle cells. CircRNA-284 primers align divergently to the circRNA-284 host gene (Homeodomain Interacting Protein Kinase 3, HIPK3), but yield a product when circRNA-284 is present. As a control, a pair of primers that align convergently to the host gene (Convergent) were also used in parallel and produce the expected product.



Supplementary Figure III. Extracellular microparticles isolated from cultured VSMCs contain miR-221 and circR-284. Human aortic vascular smooth muscle cells were incubated in Dulbecco's Modified Eagle Medium supplemented with 10% exosomes free fetal bovine serum for 48 hours. Extracellular microparticles were prepared using the ExoQuick-TC Solution (System Biosciences, Inc.) according to the manufacturer's instructions. Total RNA was prepared and droplet digital PCR was performed as described to measure the miR-221 and circR-284 content of the microparticles.

Supplementary Tables

Supplemental Table I : Primer Assays

Name	Qiagen Cat#
miR221	ms00003857
MiR222	ms00007609
MiR145	ms00003528

Supplementary Table II. Fold Change in Categorical Variables in the Discovery Study

Group	Fold Change	95 % CI	p-value
Male	1.97	0.40 - 9.72	0.40
Smoker	2.37	0.49 - 11.61	0.28
African American	0.91	0.06 - 14.07	0.94
Statin Use	0.58	0.13 - 2.65	0.47
Anti-Coagulant Use	0.23	0.05 - 0.99	0.05
Anti-Platelet Use	0.42	0.10 - 1.80	0.24
Chronic Kidney Disease	1.40	0.21 - 9.34	0.72

Supplementary Table III. Correlation between serum circR-284:miR-221 and continuous variables in the Discovery Study

Variable	R	p-value
Total Cholesterol	-.132	.496
High Density Lipoprotein	-.088	.650
Low Density Lipoprotein	-.135	.484
Triglycerides	-.111	.565
Serum Creatinine	.144	.369
Age	.194	.224
Body Mass Index	.065	.685

Supplementary Table IV. Fold Change in Categorical Variables in the Validation Study

Group	Mean Difference	95 % CI	p-value
Male	-0.91	-1.83 - 0.01	0.05
Smoker	0.31	-0.62 - 1.24	0.51
Statin Use	0.82	-0.43 - 2.06	0.19
Anti-Coagulant Use	0.12	-0.99 - 1.23	0.83
Anti-Platelet Use	0.21	-0.71 - 1.13	0.65

Supplementary Table V. Correlation between serum circR-284:miR-221 and continuous variables in the Validation Study

Variable	R	p-value
Total Cholesterol	.074	.494
High Density Lipoprotein	.040	.051
Low Density Lipoprotein	.121	.257
Triglycerides	.152	.147
Serum Creatinine	.058	.544
Age	.214	.023
Body Mass Index	-.046	.633

Supplementary Table VI. Association of Cardiovascular Risk Factors and Recent Cerebrovascular Event

Risk Factor	Odds Ratio (95% CI)		p-value
Age, years	1.04	(0.88,1.22)	0.64
Gender, Male	0.50	(0.03,8.76)	0.64
Total Cholesterol, mg/dL	0.84	(0.25,2.79)	0.78
HDL, mg/dL	1.26	(0.37,4.3)	0.71
LDL, mg/dL	1.15	(0.35,3.81)	0.82
Triglyrides, mg/dL	1.05	(0.82,1.34)	0.71
Body Mass Index, kg/m^2	1.14	(0.86,1.5)	0.36
Hypertension	0.01	(0,14.16)	0.22
History of Smoking	3.09	(0.09,107.56)	0.53
Constant	0.00	(0,183.49)	0.14
Serum circR-284:miR-221	234.01	(3.43,15955.97)	0.01

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2. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Loewer A, Ziebold U, Landthaler M, Kocks C, le Noble F, Rajewsky N. Circular rnas are a large class of animal rnas with regulatory potency. *Nature*. 2013;495:333-338