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MicroRNA 320a Predicts Chronic Axial and Widespread Pain Development Following Motor Vehicle Collision in a Stress-Dependent Manner

STUDY DESIGN: Prospective human cohort study combined with molecular studies.

BACKGROUND: A microRNA is a small, noncoding RNA molecule that can play a role in disease onset. Recent studies found that circulating levels of microRNA 320a (miR-320a) are associated with musculoskeletal pain conditions and that miR-320a is stress responsive.

OBJECTIVES: To investigate whether circulating expression levels of miR-320a in the peritraumatic period predict persistent axial musculoskeletal pain 6 months after motor vehicle collision (MVC).

METHODS: We evaluated whether (1) circulating miR-320a and related members of the miR-320a family predict axial musculoskeletal pain and other musculoskeletal pain outcomes 6 months following MVC, and (2) miR-320a regulates stress system and pain-related transcripts in cell culture. Given the wealth of data suggesting that biological mechanisms influencing pain outcomes are often sex and/or stress dependent, interactions between miR-320a, stress, and sex were evaluated.

RESULTS: In primary analyses (n = 69), a significant crossover interaction was observed between the influence of circulating miR-320a and peritraumatic distress (β = −0.01, P = .002) on post-MVC axial musculoskeletal pain. Reduced peritraumatic miR-320a expression levels predicted axial musculoskeletal pain in distressed individuals (β = −0.12, P = .006) but not nondistressed individuals. In secondary analyses, miR-320a predicted widespread musculoskeletal pain, and related members of the miR-320a family also predicted axial and widespread musculoskeletal pain. In cell culture, miR-320a bound stress and pain-associated 3'UTR transcripts (FKBP5, ADCYAP1, PER2, and NR3C1).

CONCLUSION: These data suggest that miR-320a may help mediate regional and widespread changes in pain sensitivity after MVC. *J Orthop Sports Phys Ther 2016;46(10):911-919. doi:10.2519/jospt.2016.6944*

KEY WORDS: *cervical spine, WAD, whiplash*

Physical therapists play a central role in the provision of active management strategies (eg, range of motion and exercise) to prevent the development of chronic musculoskeletal pain (MSP) after traumatic events, such as a motor vehicle collision (MVC). However, despite the hypothesized benefit of such strategies, the results of randomized controlled trials during the past decade indicate that they have, at most, modest effects.[26,44,51] During the same period, evidence that neurological/stress/immune processes play an important role in post-MVC pain pathogenesis has continued to accrue.[34,38,50] These data suggest that multimodal interventions that combine active management strategies with pharmacologic/molecular interven-

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tions targeting such processes may be more effective than active management strategies alone. Such multimodal interventions represent a potential paradigm of future care.

Examples of neurological/stress/immune processes contributing to chronic post-MVC MSP development that might be targeted by such pharmacologic/molecular interventions have been identified in animal studies.[21,27,29] These investigations have demonstrated that animals exposed to several sessions of unpredictable sound stress show persistently elevated levels of circulating stress hormones (eg, catecholamines and glucocorticoids).[27,28] These elevated stress hormones in turn sensitize primary afferent nerves throughout the body and cause previously nonpainful sensations to become painful.[13,27]

While such animal work provides an important example of how stressful events alone can cause chronic pain, specific understanding of the molecular mediators involved in the pathogenesis of post-MVC pain in humans is needed. One specific type of molecular mediator that may contribute to changes in neurosensory processing after MVC is microRNA (miRNA). A small, noncoding RNA molecule, miRNA regulates gene expression by binding target messenger RNA (mRNA) and preventing translation[1] (**APPENDIX A FIGURE 1**, available at www.jospt.org). The study of miRNA has led to important new knowledge regarding the pathogenesis of many common diseases[23,43] and may advance our understanding of chronic post-MVC MSP pathogenesis toward identifying and targeting molecular mediators to reduce activity- and movement-related pain.

In this study, we evaluated whether circulating blood levels of a candidate molecular mediator, microRNA 320a (miR-320a), in the hours after MVC would predict post-MVC axial MSP (AP) outcomes. We chose to study miR-320a because recent studies have found that circulating levels of miR-320a are associated with other MSP conditions,[2,12,46,48] cell culture studies indicate that miR-320a expression is modulated by stress,[33] and in silico analyses suggest that miR-320a may regulate genes involved in stress system function. We hypothesized that circulating levels of miR-320a and related members of the miR-320a family in the hours after MVC would predict post-MVC AP outcomes at 6 months. In secondary analyses, we also evaluated whether miR-320a predicted widespread musculoskeletal pain (WP) or fibromyalgia (FM) 6 months after MVC. We hope to highlight potential neurobiological mechanisms contributing to post-MVC MSP and the potential value of combining physical therapy and molecular interventions.

METHODS

Study Design, Setting, and Participants

This prospective longitudinal study enrolled African American individuals who presented within 24 hours of MVC to 1 of 8 emergency departments (EDs) in 3 states (Michigan, Pennsylvania, and Florida) between July 2012 and July 2013. The study enrolled African Americans because of the pressing need for pain studies that focus on such understudied, high-risk groups.[9,11,17,37,49] Individuals between 18 and 65 years of age presenting to the ED within 24 hours of MVC, who did not have fracture or other injury requiring hospital admission, were screened for eligibility. Patients who were not alert and oriented were excluded, as were patients who did not self-identify as African American, were pregnant, imprisoned, unable to read and understand English, or taking opioids above a total daily dose of 30 mg of oral morphine or the equivalent. The study protocol was approved by the Institutional Review Board at The University of North Carolina at Chapel Hill and was also approved by the Institutional Review Board of each participating hospital (UF Health Jacksonville, Jacksonville, FL; Henry Ford Hospital, Detroit, MI; Sinai-Grace Hospital, Detroit, MI; Albert Einstein Medical Center, Philadelphia, PA; Detroit Receiving Hospital, Detroit, MI; St Joseph Mercy Ann Arbor Hospital, Ypsilanti, MI; Spectrum Health Butterworth Hospital, Grand Rapids, MI; and William Beaumont Hospital, Royal Oak, MI). All participants provided written informed consent before enrollment.

Study Procedures

Eligible and consenting participants provided a blood sample in the ED and completed an ED interview evaluation. Research assistants performed interview evaluations at the time of the ED visit using a web-based survey with explicit definitions of variables. Participant demographic characteristics (including age, sex, income, height, weight, and educational attainment) were obtained from the ED medical record and from participant self-report.

Before enrolling patients in the ED, each research assistant completed a study-training module followed by an interview with a standardized, mock-ED patient. Injury characteristics and medications administered in the ED were obtained by data extraction from the ED medical record. Six months after the MVC, participants completed a follow-up interview by telephone, online, or mail. Participants were compensated $75 for completing the ED protocol and $55 for completing the 6-month interview.

Assessments and Outcome Definitions

The MVC-related MSP intensity and distribution in the past week were assessed in the ED and at 6 months using the modified regional pain scale.[54] The MSP intensity in each region was evaluated via a 0-to-10 numeric rating scale (NRS), with 0 as no pain and 10 as the maximum possible pain.[15] Axial musculoskeletal pain, the most common and most disabling post-MVC MSP,[4] was defined by the axial body region (neck, left and right shoulder, upper and lower back) with the highest reported MSP. For the dichotomous AP variable, individuals reporting an MSP severity of 7 or greater in at

least 1 axial body region (the neck, upper back, lower back, left shoulder, or right shoulder) were defined as having severe AP.[16,31] Other less common but disabling outcomes of MVC include WP[24,25] and FM.[7,40] Widespread MSP was defined according to American College of Rheumatology 1990 criteria.[55] Fibromyalgia-like symptoms 6 months after MVC were defined by the annotated American College of Rheumatology criteria, together with a fatigue severity score of 6 or greater (0-10 NRS) in the past week.[40]

Distress in the ED was measured using the Peritraumatic Distress Inventory, a 13-item questionnaire assessing the level of distress experienced immediately after a traumatic event.[6] Each item on the questionnaire was evaluated via an NRS ranging from 0 (no distress) to 4 (high distress). A cutoff of 23 was used to identify those with substantial distress.[45]

RNA Collection and Isolation

Research assistants collected blood samples in the ED at the time of enrollment, using PAXgene RNA tubes. Total RNA (including miRNA) was isolated using the PAXgene blood miRNA kit (QIAGEN) and stored at –80°C until use. RNA concentration and purity were measured using a NanoDrop 1000 (Nanodrop Technologies, Wilmington, DE) and RNA integrity was measured using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Molecular Methods

Template libraries for miRNA sequencing were produced as described previously using an adaptation of published protocols.[35,47] Twelve barcoded libraries were combined per lane and sequenced on a HiSeq 2000 (Illumina, San Diego, CA). Raw sequence reads were processed using a custom bioinformatics pipeline, as described previously.[35] The stem-loop RT-qPCR method described by Chen et al[10] was used to validate sequencing results. Stem-loop RT primers and TaqMan probes for miR-320a were obtained from Life Technologies (Carlsbad, CA). MicroRNA-320a expression was normalized to RNU48, and RT-qPCR validation of sequencing was performed on a random subset of the study participants (due to limited quantities of participant RNA). Lentiviral constructs containing either a firefly luciferase gene (pL-SV40-GL3) or a renilla luciferase gene (pL-SV40-RLUC) were used for dual-luciferase assays.[20] The 3'UTRs of human genes FKBP5, PTGER3, ADCYAP1, NR3C1, and PER2 were amplified from human cell line genomic DNA, using primers as indicated in **APPENDIX B TABLE 1** (available at www.jospt.org). The amplified 3'UTRs consisted of either the entire 3'UTR or a portion thereof, which preserved the relative location of the miR-320a binding site in the context of the full-length 3'UTR. The resulting PCR products were cloned downstream of the firefly luciferase gene in pL-SV40-GL3, using XhoI and NotI or EcoR1 restriction enzyme sites. These newly created constructs were then mutated at the predicted miR-320a binding sites, such that 2 to 3 mismatches were incorporated into the seed-binding region (primers in **APPENDIX B TABLE 1**). To determine mRNA transcripts that might be regulated by miR-320a, we used Target Scan Release 6.0 (Whitehead Institute for Biomedical Research, Cambridge, MA) to obtain all of the predicted targets for the miR-320abcd/4429 conserved miRNA family. Then, to determine whether any of the predicted miR-320a targets were established pain genes, we compared the predicted target list to a list of all identified pain genes catalogued by PainNetworks (downloaded August 2014, http://www.painnetworks.org). To determine whether any of the predicted miR-320a targets were established stress system genes, we compared the predicted target list to a list of stress-associated genes (**APPENDIX B TABLE 2**). HEK293T cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS) and 1% gentamicin. Binding of miR-320a to each 3'UTR transcript was assessed using the above described GL3- (FLUC) and RLUC-based indicator vectors. HEK293T cells were cotransfected with 120 fmol of pC-320a, 5 fmol of pL-SV40-RLUC, and 20 fmol of the indicated pL-GL3 construct (or empty pL-GL3 control), using Fugene 6 Transfection Reagent (Promega Corporation, Madison, WI). Forty-eight hours after incubation at 37°C, 5% CO_2, cells were collected and lysed. RLUC and FLUC levels were measured on a SpectraMax microplate reader (Molecular Devices, LLC, Sunny-

TABLE 1 Baseline Characteristics of Study Participants

Characteristic	Value
Participants, n	69
Age, y*	37 ± 12
Sex (female), n (%)	40 (58)
Education, n (%)	
High school or less	21 (31)
Some college	34 (50)
College	11 (16)
Postcollege	2 (3)
Body mass index, kg/m^2*	30 ± 7
Median time from MVC to ED, min	67
Substantial peritraumatic distress, n (%)	31 (45)
ED pain severity (0-10 NRS)*	7 ± 2

Abbreviations: ED, emergency department; MVC, motor vehicle collision; NRS, numeric rating scale.
**Values are mean ± SD.*

vale, CA), using substrates from a dual-luciferase reporter assay kit, as described (Promega), and FLUC values were normalized to RLUC values.

Statistical Analysis

General linear models of AP scores at 6 months were used to test for main effects and sex-miR-320a and stress-miR-320a interactions, adjusting for age and ED study site. Sex- and stress-dependent effects were evaluated based on evidence that such interactions are frequently present and important.[3,42,53] To derive odds ratios (ORs) and test associations between miR-320a and dichotomous outcomes, logistic regression models adjusting for age, sex, and ED study site were used. Differences in ED miR-320 family member expression between those who did and did not subsequently develop AP, WP, or FM at 6 months were evaluated by comparing mean expression levels in the 2 groups, with negative values indicating lower expression in individuals developing MSP. Statistical analyses were carried out using SPSS Version 21.0 (IBM Corporation, Armonk, NY) or SAS University Edition (SAS Institute Inc, Cary, NC).

RESULTS

Cohort

Samples were drawn randomly from a large prospective cohort study of African Americans (R01AR060852). Characteristics of the study sample (n = 69) are shown in **TABLE 1**. All individuals in this study presented to the ED between 2 and 10 hours following an MVC. Nearly 6 in 10 were female. Most (60%) were less than 40 years of age, had some college education, and were overweight (body mass index [BMI] greater than 25 kg/m^2). Only individuals who were discharged from the ED to home and reported no lacerations, avulsions, or major tissue injury were included. All participants had an Abbreviated Injury Scale[19] severity score of 1, indicating minor injury.

TABLE 2 General Linear Model Examining the Relationship Between Candidate Predictors and Axial Pain Outcome 6 Months After Motor Vehicle Collision (n = 68)

Variable*	F	β	SE	*P* Value
Age	0.99	0.04	0.04	.323
Sex	3.61	2.39	1.26	.063
Distress†	3.93	0.11	0.05	.053
microRNA 320a‡	5.98	0.16	0.06	.018
Sex	3.19	−0.09	0.05	.080
Distress	10.30	−0.01	0.002	.002

Abbreviation: SE, standard error.
**Study site was also included as a categorical variable.*
†Assessed in the emergency department using the Peritraumatic Distress Inventory (0-10).
‡Expression levels were measured from blood samples collected in the emergency department.

Initial Linear Regression Model of Persistent AP Severity 6 Months Following MVC

In an initial linear regression model of candidate predictors, the interaction of miR-320a and peritraumatic distress strongly predicted AP presence/severity 6 months after MVC (β = −0.01, P = .002) (**TABLE 2**). This suggested a cross-over interaction and indicated the need to evaluate the influence of miR-320a among those who did and did not experience substantial peritraumatic distress separately.

Circulating miR-320a Predicts AP Presence/Severity Among Individuals Experiencing Substantial Peritraumatic Distress Among individuals who experienced substantial peritraumatic distress after MVC, ED blood miR-320a levels in the hours after MVC strongly predicted AP presence/severity at 6 months (β = −0.12, P = .006) (**TABLE 3**). In contrast, among those without substantial peritraumatic distress, miR-320a expression levels did not predict AP at 6 months, with a point estimate for miR-320a influence near zero (**TABLE 3**). This marked interaction between miR-320a and peritraumatic distress and AP outcome is shown in **FIGURE 1**. This direction of effect observed from sequencing analyses was validated using RT-qPCR analysis in a subset of individuals (data not shown).

miR-320a Expression Levels Predict WP Among Individuals Experiencing Substantial Peritraumatic Distress We also evaluated whether miR-320a predicted other adverse MSP outcomes after MVC, including WP and FM. Low levels of miR-320a expression predicted WP in individuals who were experiencing substantial peritraumatic distress (OR = 0.775, P = .037), but not in those without substantial peritraumatic distress (OR = 0.837, P = .122). As with AP, lower levels of miR-320a predicted WP. Expression levels of miR-320a did not predict the development of FM in those with or without peritraumatic distress (with distress: OR = 0.978, P = .733; without distress: OR = 0.045, P = .321).

Additional Members of the miR-320 Family Predict Post-MVC MSP Outcomes Previous reports have shown that miRNA from the same family can downregulate mRNA targets in an additive fashion.[22,52] Therefore, we analyzed whether any additional members of the miR-320 family predict AP and/or WP and found that miRNA 320bc and miR-320a 5 prime isomiRs significantly predict AP development in distressed individuals (P<.05 or P<.10 at the trend level) (**APPENDIX B TABLE 3**). Some miR-320 family members also predicted WP (**APPENDIX B TABLE 3**).

Bioinformatics Analyses Predict That

TABLE 3 Linear Regression Models Examining the Relationship Between microRNA-320a Expression and Axial Pain Severity 6 Months After Motor Vehicle Collision Among Individuals With and Without Substantial Distress in the Emergency Department

	With Distress (n = 31)*				Without Distress (n = 37)			
Variable	**F**	**β**	**SE**	***P* Value**	**F**	**β**	**SE**	***P* Value**
microRNA 320a	8.71	−0.12	0.04	.006	0.083	0.01	0.02	.774
Age	0.92	0.05	0.05	.347	0.003	−0.003	0.06	.955
Sex	2.69	2.00	1.22	.112	0.027	−0.22	1.34	.871

Abbreviation: SE, standard error.
**Distressed individuals were defined by a score of 23 or higher on the Peritraumatic Distress Inventory, as assessed in the emergency department upon enrollment into the study.*

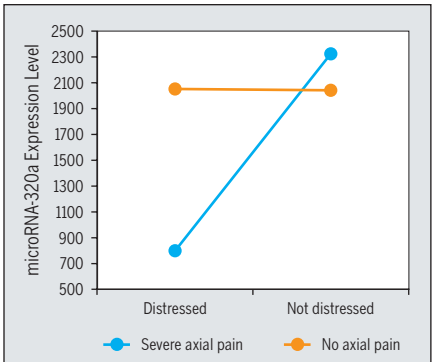

FIGURE 1. Distressed individuals with lower levels of microRNA 320a had increased risk of developing severe axial pain 6 months following motor vehicle collision than did distressed individuals with higher levels of microRNA 320a. Nondistressed individuals with higher levels of microRNA 320a had the same risk of developing persistent axial pain versus recovering (no axial pain) following motor vehicle collision.

miR-320a Regulates Important Mediators of the Stress-MSP Axis As described above, a primary mechanism by which miRNA affects physiology is by binding to mRNA and preventing its translation[1] (**APPENDIX A FIGURE 1**). In order to determine potential miR-320a mRNA targets that could affect chronic MSP outcomes, we assessed for predicted targets known to be involved in the response to stress and/or to MSP transduction. Using bioinformatics analyses (see Methods), a number of candidate genes were identified (**APPENDIX B TABLE 2**), including targets known to influence both stress and MSP-related outcomes: PTGER3, ADCYAP1, NR3C1, PER2, and FKBP5 (**APPENDIX A FIGURE 2**).

FKBP5, ADCYAP1, NR3C1, and PER2 Are Direct Targets of miR-320a The above genes were identified as potential miR-320a targets, based on DNA sequence and predicted binding (in silico). We next evaluated whether this was the case in cell culture (in vitro). Using dual-luciferase reporter assays, we found that 4 of 5 predicted target genes were directly bound by miR-320a, including FKBP5, ADCYAP1, NR3C1, and PER2 (**FIGURE 2**). In addition, providing further evidence of direct binding of miR-320a to these 4 genes, disruption of the predicted seed-binding region of miR-320a in each of the 3'UTRs of these genes inhibited miR-320a binding.

DISCUSSION

In this study, blood expression levels of miR-320a in the early hours after MVC predicted AP and WP outcomes at 6 months. Interestingly, a strong interaction between miR-320a and peritraumatic distress was observed, such that miR-320a influenced post-MVC MSP outcomes most strongly among individuals who experienced substantial peritraumatic distress (45% of the current sample). Supporting the validity of this finding, additional members of the miRNA-320 family also predicted AP and WP outcomes among those with substantial distress, and both bioinformatics analyses and subsequent in vitro studies have indicated that miR-320a modulates the translation of a number of genes critical to the physiologic response to stress and to MSP processing. These data add to evidence that the pathogenesis of chronic post-MVC AP is shaped by interactions between nervous, stress, and immune systems. In addition, these data identify a potential molecular mediator of chronic post-MVC AP.

The fact that miR-320a expression level strongly interacted with level of participant distress has several important ramifications. First, these data are consistent with increasing data that physiologic systems involved in the body's response to a life threat play an important role in the pathogenesis of chronic MSP after stressful events such as MVC.[38,39] Second, these data suggest that the molecular pathways/mediators that result in chronic AP after MVC are not homogeneous within this population, but may vary substantially from individual to individual. Such a concept has long seemed logical clinically, but molecular data demonstrating dependencies between specific molecular factors and specific environmental factors (eg, substantial peritraumatic stress, a more stressful living environment in the months after MVC[53]) and/or specific individual factors (eg, sex[3]) have only been demonstrated more recently. Similarly, these data suggest that the optimal combination of multimodal interventions to prevent and treat post-MVC AP and other MSP may vary from patient to patient. The identification of novel, effective preventive treatment interventions to combine with physical therapy and other interventions, and the targeting of the right treatment or combination of treatments to the right individual, at the right time, constitutes the major challenge for the field in the 21st century.

Whether miRNA is a marker for other pathogenic processes directly involved in

chronic pain pathogenesis after MVC or is itself directly involved in chronic pain pathogenesis is unknown. If miR-320a is directly involved, then lower expression levels of miR-320a could result in increased expression of key transcripts involved in MSP pathogenesis. For example, as demonstrated above, miR-320a binds the FKBP5 transcript, thus a reduced level of miR-320a would be anticipated to result in greater cellular levels of FKBP5. Evidence suggests that increased levels of FKBP5 increase vulnerability to posttraumatic sequelae, including both chronic MSP[5,36] and posttraumatic stress disorder.[30,41] Importantly, this example describes the binding of miR-320a to only a single gene target, FKBP5, whereas (as described above) miR-320a targets a number of gene targets. The overall net effect of changing miR-320a levels across all of its transcriptional targets is an important topic of future study.

We examined AP as our primary outcome in this study, not only because it is a theme of this special issue, but also because it is the most common and morbid location of chronic post-MVC MSP.[4] In secondary analyses, circulating miR-320a also predicted WP 6 months following MVC in distressed individuals, but unlike the findings of a previous study,[2] it did not predict FM development. One contributing factor to this might have been the small sample size of the present study. Because FM is a relatively rare outcome after MVC, the very small number of individuals in the present analysis with FM at 6 months (n = 6) markedly reduced the statistical power of the analysis. In secondary analyses, we also examined whether miR-320 family members would predict persistent MSP following MVC. The miRNA family members originate from different regions of the genome but share overlapping sequences, most notably in the seed-binding region of an miRNA (the "seed" of an miRNA includes nucleotides 2-8 of the 21-23nt miRNA molecule[18]). Because the major determining factor for whether an miRNA binds to its target depends on nucleotide complementarity between the seed region of an miRNA and its target, family members often share and cooperatively bind targets.[1,22,52] Therefore, the fact that additional miR-320 family members predict AP and WP in distressed individuals indicates that those family members might also bind the same MSP and stress-relevant targets, thus supporting, and potentially magnifying, the effect of miR-320a. In addition to miR-320 family members, we also examined whether 5'isomiRs of miR-320a could predict persistent MSP development following MVC. Sequence variants of mature canonical miRNA (isomiRs) can vary at the 5' and 3' ends of the molecule. We examined 5' variants because the addition (+) or subtraction (−) of a nucleotide to the 5' end will change the seed sequence and thus the targeting of the miRNA. The miR-320a+1 significantly predicted WP development in this study. Because multiple similar miRNA were shown to predict post-MVC MSP development and might be involved in MSP pathogenesis, any potential molecular therapeutics that target miR-320a would benefit from overlapping efficacy against related family members and isomiRs.

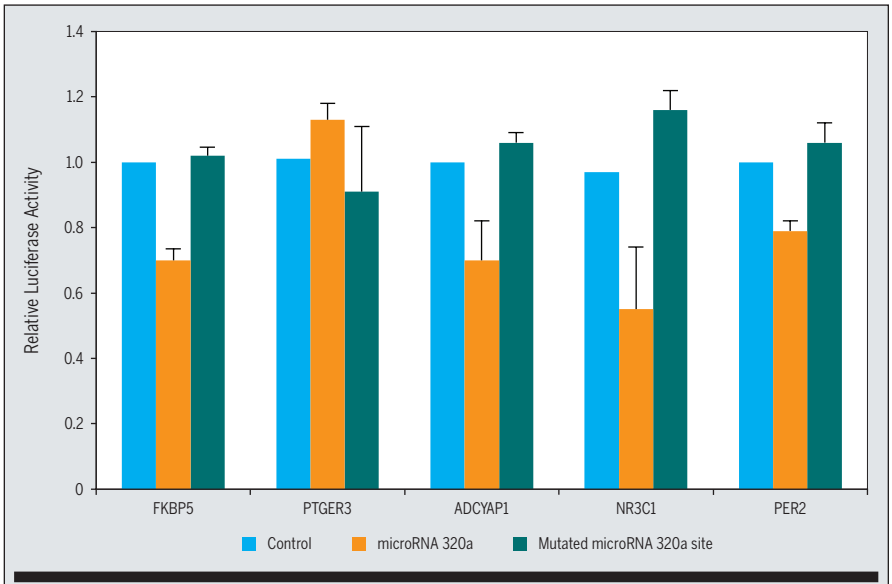

FIGURE 2. Determination of pain and stress-relevant messenger RNA targets of microRNA 320a. Five gene transcripts were determined to be high-confidence predicted pain/stress targets, based on whether they met the following criteria: a predicted target of microRNA 320a based on the TargetScan algorithm, a pain gene as defined by the PainNetworks database, and a stress-associated gene. Dual-luciferase assays showed that 4 of the 5 predicted targets can be bound directly by microRNA 320a (orange bars), and are repressed to varying degrees in the presence of microRNA-320a expression in HEK293T cells. microRNA-320a binding to the 4 3'UTRs was inhibited by mutation of the seed-binding site (green bars). Error bars represent the standard deviation from 3 independent experiments. Abbreviations: HEK293T, human embryonic kidney 293 cell line containing the SV40 T-antigen; 3'UTR, 3' untranslated region.

Some limitations should be considered when interpreting the results of this study. First, the sample size of this study was relatively small. Future studies with larger samples and greater power are needed to fully evaluate the influence of miR-320a and other miRNA on post-MVC MSP outcomes and to evaluate whether other genetic interactions (such as one between miR-320a and participant sex, as is suggested by the current results) play a role in predicting chronic pain outcomes. Second, we do not know how expression levels of miR-320a changed over time after MVC. Further studies evaluating miRNA trajectories and the influence of the trajectory intercept and slope on post-MVC outcomes would also be valuable. For

TABLE 4 List of Abbreviations

3'UTR	3 prime untranslated region
AP	axial musculoskeletal pain
ED	emergency department
FM	fibromyalgia
GL3 (FLUC)	firefly luciferase gene
HEK293T	human embryonic kidney 293 cell line containing the SV40 T-antigen
miR-320a	microRNA 320a
miRNA	microRNA
mRNA	messenger RNA
MSP	musculoskeletal pain
MVC	motor vehicle collision
NRS	numeric rating scale
qPCR	quantitative polymerase chain reaction
RLUC	renilla luciferase gene
RT	reverse transcription
WP	widespread pain

example, future investigations could examine whether the peritraumatic period represents a distinct predictive window or miRNA levels in the early aftermath of MVC are representative of miRNA levels over time after MVC. Third, the targets of miR-320a identified in this analysis represent only a subset of all miR-320a targets, and the potential molecular mediators of miRNA, or net cellular influence of miRNA, that result in the association between this miRNA and chronic MSP development are not known. Fourth, miRNA expression following MVC was assessed in whole blood samples. Whole blood is both feasible and ethical to collect from human cohort studies; however, the relevance to MSP and stress biology has not been studied extensively. Importantly, tissue-specific expression analyses from animal studies (data not shown) demonstrate that blood and neuronal tissues share overlapping miR-320a expression. Finally, our study population was limited to African Americans, an understudied group that has been shown to experience an increased burden of adverse MSP outcomes after trauma.[8,9,14,32] The generalizability of our findings to other ethnic groups is unknown.

CONCLUSION

The results of this study show that peritraumatic miR-320a expression levels predict persistent post-MVC MSP among individuals experiencing substantial peritraumatic distress. Combined with animal data showing tissue-specific expression of miR-320a in stress and pain-relevant tissues, and molecular data showing that miR-320a can downregulate gene transcripts involved in stress and MSP signaling, the results of this study implicate miR-320a in the pathogenesis of persistent post-MVC MSP. These preliminary data support the potential value of elucidating such mechanisms and mediators, and suggest that interventions targeting such mechanisms and mediators have exciting potential as multimodal adjuvants to physical therapy care. A list of the abbreviations used in this article may be found in **TABLE 4**.

KEY POINTS

FINDINGS: MicroRNA 320a binds gene transcripts involved in pain and stress signaling and predicts persistent AP and WP 6 months after MVC.

IMPLICATIONS: These data add to evidence that the pathogenesis of persistent post-MVC pain is shaped by interactions between nervous, stress, and immune systems, and identify a specific potential molecular mediator of persistent post-MVC pain. Pharmacologic/molecular interventions targeting such mechanisms have exciting potential as adjuvants to traditional physical therapy care.

CAUTION: Our results are based on data from a single small cohort, thus the data should be replicated/validated in a larger and/or independent MVC cohort.

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APPENDIX A

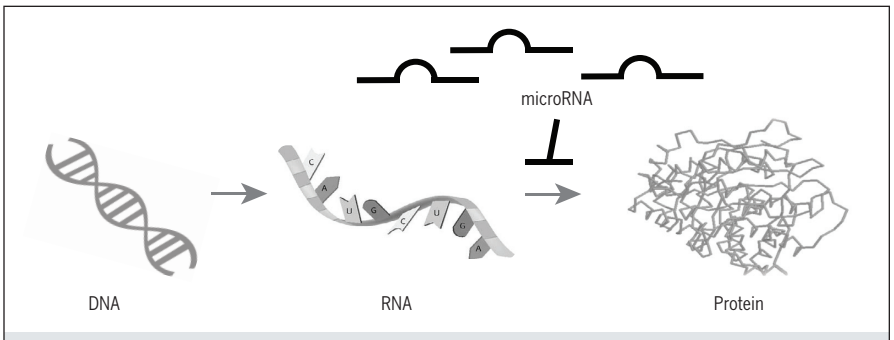

FIGURE 1. Normally, DNA is transcribed into RNA, and RNA is translated into protein (gray, left to right). microRNA (black, top) are small, noncoding RNA molecules that regulate gene expression by binding target messenger RNA and preventing gene translation into protein.

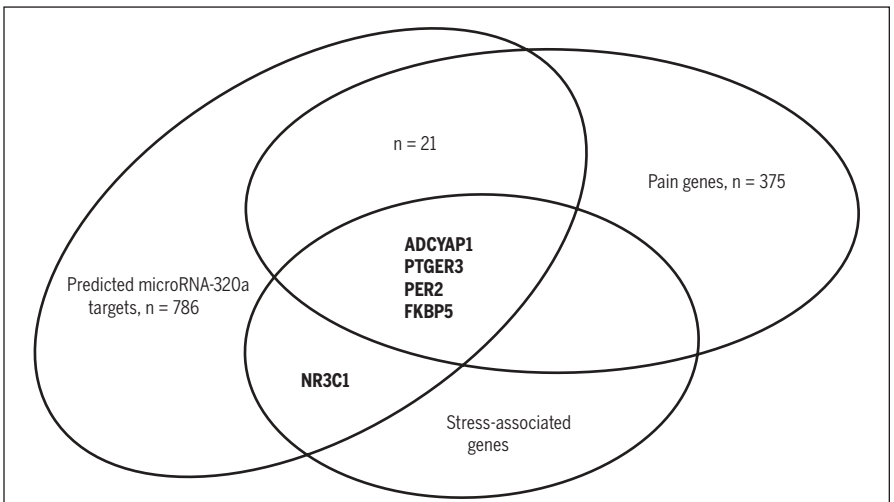

FIGURE 2. Pain and stress-associated targets of microRNA 320a were determined by examining overlap between predicted targets of microRNA 320a (identified via TargetScan Version 6.0) and established pain and stress genes. Overlap was found for ADCYAP1, PTGER3, PER2, FKBP5, and NR3C1.

APPENDIX B

TABLE 1 Primers Used in the Study

Primer	Sequence	Construct
FKBP-F	atgatgCTCGAGGCCACGCCAAGGAGGGAAGAGTCC	pL-GL3-FKBP5
FKBP-R	atgatgGAATTCGGATTGCCAAGTCAGCATATGAGG	pL-GL3-FKBP5
ADCY-F	catgacCTCGAGAAGAGTGAGGTAAGCAAGCTCC	pL-GL3-ADCYAP1
ADCY-R	catgacGCGGCCGCTAAAATGCTTGCAACTAGCCACGGG	pL-GL3-ADCYAP1
PER2-F	catgacCTCGAGAATTTTGTCCTCTCTTGGGGACTGC	pL-GL3-PER2
PER2-R	catgacGCGGCCGCTGAGGTTTTGTAAAAAGCCGGGCAG	pL-GL3-PER2
PTG3-F	catgacCTCGAGTAGCAAATGCTGTCTCCAGCTGCTC	pL-GL3-PTGER3
PTG3-R	catgacGCGGCCGCGTCAAAGACTGGGGAACACTTTTGGTG	pL-GL3-PTGER3
NR3-F	catgacCTCGAGTAGTAGCCCTTTCTGTGTGCACCTTACC	pL-GL3-NR3C1
NR3-R	catgacGCGGCCGCTGCTGGTACCTCTATGCAAACTATTGCC	pL-GL3-NR3C1
F5mut-F	CCAUGCCGGCUUUUUUGUCUUUUUAAGAUUAUUGGC	pL-GL3-FKBP5-320mut
F5mut-R	CAAAAAAGCCGGCATGGATCCTAAATGAC	pL-GL3-FKBP5-320mut
ADCmut-F	TTTGGAATTCTAGTGCCTTTCTTCGCC	pL-GL3-ADCY-320mut
ADCmut-R	ACTAGAATTCCAAACTGCATCCCGTG	pL-GL3-ADCY-320mut
PERmut-F	CCAAGAATTCTGGCTTCTGGTGTGG	pL-GL3-PER2-320mut
PERmut-R	GCCAGAATTCTTGGTTTGCCAAACAACG	pL-GL3-PER2-320mut
PTGmut-F	TGCAGAATTCTTGGTAACAAATATCGCTAAACC	pL-GL3-PTGER-320mut
PTGmut-R	CCAGAATTCTGCACATGCAAGTTAAGTG	pL-GL3-PTGER-320mut
NR3mut-F	ACTGGAATTCTACATGCAATTTATTAAAATGATTG	pL-GL3-NR3C1-320mut
NR3mut-R	TGTAGAATTCCAGTAGCCCTTCCCTTC	pL-GL3-NR3C1-320mut

TABLE 2 Stress System Genes Used for microRNA-320a Target Prediction Analyses

HPA Axis	Catecholaminergic	Circadian Rhythm
ADCYAP1	ADRA1A	ARNTL
CCKBR	ADRA1B	BHLHB2
CLPS	ADRA2A	CCK
CPS1	ADRA2B	CCKAR
CRH	ADRA2C	CLOCK
CRHBP	ADRB2	HCRTR1
CRHR1	ADRBK2	NPAS2
CRHR2	ARRB2	PER2
FKBP5	COMT	PER3
NR3C1	SLC6A2	RORA
NR3C2	TH	TIMELESS
PTGER3	...	...

Abbreviation: HPA, hypothalamic-pituitary-adrenal.

APPENDIX B

TABLE 3 Evaluation of Circulating Expression Levels of miR-320 Family Members in Distressed Individuals* Who Develop Persistent Pain Outcomes at 6 Months Versus Individuals Who Recover Following Motor Vehicle Collision (n = 35)

	Axial Pain			Widespread Pain			Fibromyalgia		
miR	Fold Change	Sequence Reads (Pain/No Pain)	*P* Value†	Fold Change	Sequence Reads (Pain/No Pain)	*P* Value†	Fold Change	Sequence Reads (Pain/No Pain)	*P* Value†
miR-320a	−2.6	797/2053	.030	−3.0	645/1942	.037	−1.9	862/1626	.733
miR-320a+1	−2.2	6.2/13.4	.108	−3.5	3.9/13.6	.033	−6.6	1.8/11.8	.360
miR-320a−1	−2.4	38.1/90.5	.067	−2.7	32.0/85.8	.059	−1.6	44.1/69.7	.898
miR-320b	−2.7	5.0/13.7	.046	−3.3	3.9/12.9	.038	−1.6	6.5/10.6	.910
miR-320c	−2.0	4.1/8.2	.051	−2.0	3.9/7.7	.100	−1.1	6.5/7.2	.713
miR-320d	−2.3	2.1/4.8	.163	−2.6	1.8/4.6	.312	−1.1	3.7/4.1	.727

Abbreviation: miR, microRNA.
**Distressed individuals were defined by a score of 23 or higher on the Peritraumatic Distress Inventory, as assessed in the emergency department upon enrollment into the study.*
†P values were determined using logistic regression models adjusted for age, sex, and emergency department study site.