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Angiotensin Receptor Type 1 Single Nucleotide Polymorphism A1166C is Associated with Malignant Arrhythmias and Altered Circulating miR-155 Levels in Patients with Chronic Heart Failure

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Abstract

Background—Sudden cardiac death (SCD) due to ventricular tachyarrhythmias accounts for approximately 450,000 annual deaths in the U.S.; many of these cases involve patients with chronic heart failure (HF). Prediction of which HF patients are most susceptible to SCD is difficult, and it is uncertain whether gene polymorphisms associated with HF outcomes are also linked to arrhythmic risk.

Methods—We evaluated 485 patients with chronic HF to see whether the Angiotensin Receptor Type 1 (AT1) A1166C or Angiotensin Converting Enzyme Insertion/Deletion (ACE I/D) polymorphisms were associated with a higher rate of ventricular arrhythmias requiring implantable cardioverter defibrillator (ICD) therapies over a 5-year period. We assessed the correlation between polymorphisms and antitachycardia pacing (ATP) and/or ICD shocks.

Results—Patients with AT1-1166 CC genotype had an increased rate of all events: ATP plus ICD shocks ($p=0.02$). There was no association between ACE I/D genotype and ICD therapies. Furthermore, circulating levels of microRNA-155 (miR-155), a microRNA known to posttranscriptionally regulate AT1R expression, were significantly decreased in the CC compared to the AC and AA genotypes and were associated with ICD events.

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DISCLOSURES:

None

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Conclusion—Our study suggests that the AT1R-1166 CC genotype is associated with increased ICD therapies in patients with chronic HF, and the level of circulating miR-155 may be a potential marker for arrhythmic risk. While these findings are novel, they will need replication and validation in larger cohorts of chronic HF patients.

Keywords

Genetic Polymorphisms; Angiotensin; Angiotensin Type 1 Receptor; Sudden Cardiac Death; Heart Failure; microRNA

INTRODUCTION

Sudden cardiac death (SCD) is a leading cause of mortality in the United States, claiming as many as 462,000 lives annually (1, 2). Sudden cardiac death is most commonly related to ventricular tachyarrhythmia, and SCD occurs disproportionately in patients with chronic heart failure (HF). In fact, SCD occurs in HF patients at a rate that is 6–9 times higher than that of the general population (1). However, prediction and prevention of SCD in the individual patient remains a huge challenge for the clinician, and there is a great need for sensitive and specific markers of SCD risk that could assist in determining which individuals would most benefit from costly lifesaving therapies, such as implantable cardioverter defibrillators (ICDs) (3–5).

The renin angiotensin aldosterone system (RAAS) is activated in patients with chronic HF, and pharmacologic inhibition of angiotensin II or angiotensin type 1 (AT1) receptor have been shown to reduce SCD in patients with HF and after myocardial infarction (6, 7). In this study, we sought to prospectively determine whether polymorphisms that impact the RAAS, ACE I/D or the AT1-A1166C, were associated with ventricular tachyarrhythmias in HF patients. We studied patients who had ICDs, because this allowed us to document arrhythmic events through electrocardiograms recorded by the device. Furthermore, several recent studies have suggested that circulating microRNAs (miRNAs) could be useful as biomarkers for various human disease states, including cancer(8), acute myocardial infarction (MI) (9–12), HF(13, 14), and chronic vascular disease(15, 16). Therefore, in a subset of our study cohort, we assessed levels of circulating miRNAs that may impact the RAAS system. We hypothesized that miRNA levels in whole blood would be different in patients with the A1166C polymorphism and these changes might provide insight into why AT1-1166 CC patients are at higher risk for ventricular arrhythmias. Our data suggest that the risk of SCD in patients with HF is associated with polymorphisms in the gene encoding the AT1 receptor as well as changes in circulating levels of miR-155, a miRNA known to target the AT1 receptor (17–19).

METHODS

Study population

A cohort of 504 patients with chronic HF was recruited as part of the Genetic Risk Assessment of Defibrillator Events (GRADE) trial between 2005 and 2009. Of these 504 patients, we excluded 19 due to incomplete or inconsistent data. Thus, the analytic cohort of this study consisted of 485 subjects who were enrolled at Emory University Hospital, Emory University Hospital Midtown and the Atlanta Veterans Administration Medical Center (AVAMC). Inclusion criteria were, 1) age 18 years or older, 2) LVEF ≥ 30%, and 3) have had an ICD placed during the preceding five years or were scheduled to have an ICD placed during the week after recruitment. The Institutional Review Board of Emory University and the AVAMC's Research and Development Committee approved the study. Informed consent was obtained from all patients.

At the time of study entry, demographic information, NYHA functional class, etiology of heart disease and/or HF, family history, physical examination findings, medical therapy, and results of prior cardiac testing were collected. Detailed information for all available electrocardiograms was also recorded. Patients were followed at least annually for 5 years, as per the GRADE trial protocol. Information obtained from every clinically indicated ICD interrogation over the 5-year period was recorded with particular focus on ICD events. Follow-up data included information such as hospitalizations, medication changes, HF symptoms, palpitations, and syncope.

Commercially available transvenous ICD systems were implanted in all subjects. Defibrillator programming and choice of antiarrhythmic medications were left to the discretion of the implantation physician (cardiac electrophysiologist). Ventricular arrhythmias were analyzed from the telemetry strips, and episodes of sustained ventricular tachycardia (VT) or fibrillation (VF) were confirmed. Appropriate ICD therapies were noted. Blood samples were collected from each patient at date of enrollment for DNA analysis, and blood samples for microRNA (miRNA) expression was collected in the last year of patient enrollment in 2009.

Genotyping of the ACE I/D Polymorphism

Genomic DNA was isolated from whole blood by standard techniques utilizing the QIAamp® Blood Kit (Qiagen, Inc.). Primers 5' CTGGAGACCACTCCCAT CCTTCT 3' and 5' GATGTGGCCATCACATTCGTCAGAT 3' were designed to amplify the D and I alleles with product sizes of 382 bp and 671 bp, respectively. Polymerase chain reactions were run for 1 cycle of 93°C for 2 minutes followed by 35 cycles of 93°C for 15 seconds, 63°C for 15 seconds and 72°C for 45 seconds following a final extension of 72°C for 10 minutes using Invitrogen Platinum Taq DNA polymerase (Invitrogen Inc.). The product was identified on a 1% agarose gel stained with ethidium bromide and photographed under ultraviolet transillumination (Figure 1). Genotyping was verified by sequencing 15 subjects of the total cohort representing all three genotypes.

Genotyping of the AT1-1166 Polymorphism

Briefly, primers F: 5'-CGCCCTCAGATAATGTAA-3' and 5'-Biotin-GGCTTTGCTTTGTCTTGTG-3' were used to amplify the AT1-1166 A/C alleles with a product size of 263 bp. Polymerase chain reactions were run for 1 cycle at 94°C for 5 minutes; 45 cycles: 94°C for 30 seconds, 55 °C for 30 seconds, 72°C × 60 seconds; and 72°C for 10 minutes. Genotyping was carried out on a pyrosequencer (model PSQ96MA, Pyrosequencing, Uppsala, Sweden) following manufacturer's protocol with pyrosequencing primer 5'-CTTCACTACCAAATGAGC-3'.

Assessment of MicroRNA Expression

RNA Isolation—Venous blood samples were drawn into PAXgene Blood RNA Tubes and stored at -20°C within 24 hours before RNA extraction. The miRNA was isolated using the PreAnalytiX PAXgene miRNA isolation kit (Qiagen) according to the manufacturer's protocol.

MicroRNA qRT-PCR—MicroRNA reverse transcription was performed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) at 16°C for 30 min, 42°C for 30 min, and denaturation of the enzyme at 85°C for 5 min. The RT reaction was performed at 37°C for 1 hour followed by 5 min at 95°C. TaqMan microRNA assays (Applied Biosystems) were performed for 13 miRNAs (miR-654, miR-636, miR-218, miR-663, miR-155, miR-21, miR-10b, miR-29a, miR-200c, miR-1, miR-133, miR-208, miR-499) using the 7500 Fast Real-Time PCR System at the 9600 emulation run mode. The

7500 Fast Real Time PCR System from Applied Biosystems was used in the 7500 standard run mode, but cycle times and temperatures were changed to: 95°C-15 min; 94°C-15 s; 55°C-30 s; 70°C -30 s; 40 cycles. Ct values were converted into copy numbers ($\text{copy\#} = 2^{(-Ct)}$) and normalized to RNU48 (small nucleolar RNA).

Statistics

The genotypes associated with the polymorphisms were classified as either wild-type, heterozygous or homozygous for the variant allele. A likelihood ratio χ^2 statistic was used to test the assumption of Hardy–Weinberg equilibrium (HWE). The HWE evaluation and all other findings and statistical tests were done separately for Caucasians and African Americans. Nevertheless, overall findings and statistical tests include all subjects adjusted for race. Baseline covariates were compared by ICD therapy using the standard t test for continuous variables and the chi-squared test for categorical variables. To investigate whether the genotypes were associated with the risk of ICD therapy, odds ratios (ORs), rate ratios (RRs), and 95% confidence intervals (95% CIs) were calculated using conditional logistic regression or the Cox proportional hazards model. Circulating miRNA levels of patients with different AT1-1166 A/C alleles were compared using the Wilcoxon rank-sum test. All statistical analyses were done using the Stata statistical software program (Release 9.0, Stata Corp., College Station, TX; 2005).

RESULTS

In our study cohort of 485 patients, 186 patients (38%) had defibrillator events, classified as an appropriate shock or ATP. The mean LVEF was $20 \pm 7\%$ and 76% of patients were in NYHA class I or II. Seventy-six percent of patients were on ACE Inhibitors or ARBs, and 86% of patients on β -blockers. The demographic characteristics of the overall cohort are summarized in Table 1. Etiology of cardiomyopathy (ischemic or nonischemic), cardiovascular risk factors (arterial hypertension, hyperlipidemia or diabetes), and medications (ACE-inhibitors, ARBs, and β -blockers) were not predictive of spontaneous VT/VF events requiring ICD intervention. Also, there was no association between ICD interventions and renal function; the mean serum creatinine level for the patients without ICD events was 1.2 mg/dL (range 0.7–1.7), while the mean level for patients who had ICD interventions was 1.2 mg/dL (range 0.7 – 1.9). Subjects who received ICD therapies had a worse LVEF, 19% vs 21% ($p=0.01$) and trended to have a higher NYHA class, 2.1 vs 2.0 ($p=0.07$).

The distribution of genotype data is presented in Table 2. The genotypes for ACE I/D and AT1-1166AA were in HWE both among Caucasians and African Americans. The AT1-1166 CC genotype was prevalent in 6% of our subjects with predominance in Caucasians, which is comparable to previously reported studies (20). No association was found between the ACE I/D genotype and ICD therapies. Although the overall association between the AT1-1166 SNP and ICD therapies was not statistically significant, the odds of ICD therapy among subjects with the CC genotype was 2.5 times higher than the odds of therapy among subjects with the AA or AC genotypes ($P = 0.02$).

We also obtained the rate of ICD therapies according to genotypes for the ACE I/D and AT1-1166C polymorphisms. As with the risk analysis described above, there was no overall statistically significant association for either SNP. The rate of ICD events among subjects with the CC genotype of AT1-1166C was 20/100 per year compared with a rate of 13/100 per year among subjects with the AA or AC genotypes. Importantly, after adjusting for race, the relative rate of ICD therapies for patients having the CC genotype compared to those with the AA or AC genotypes was 1.82 (95% CI: 1.1, 3.0; $P = 0.02$).

Notably, there was no significant association between age, type of cardiomyopathy, LVEF, NYHA class, diabetes, hypertension, dyslipidemia, and medical therapy and the ACE I/D and AT1-1166 genotypes. There were no statistically significant interactions between both gene variations and ICD therapies.

MiRNAs are a recently recognized class of short (19–25 nt), single stranded, noncoding RNAs that regulate an array of cellular functions through the posttranscriptional repression of mRNAs that contain complementary sequences to the miRNA (21–25). Recent studies have shown that levels of circulating miRNAs differ in patients with HF (13, 14), suggesting that miRNA expression profiles may be valuable biomarkers for HF diagnosis and prognosis. We performed qRT-PCR on RNA isolated from whole blood of a subgroup of patients (n= 45). We assessed expression of 13 miRNAs selected based on their known or predicted association with the RAAS pathway or HF pathophysiology. MiR-636, miR-155, miR-663, miR-218, and miR-200c have known or predicted associations with the RAAS pathway (18, 26, 27). MiRNA-1, miR-21, miR-133, miR-208, miR-499, and miR-29a have been shown to be associated with cardiac remodeling and fibrosis (28–30). MiR-654 and miR-10b do not have any known association with HF pathophysiology and were included in our assessment as miRNAs whose levels we did not think would differ between our groups.

Among these 13 miRNAs, levels of miR-155 (Figure 2), miR-654, miR-663, miR-133a, miR-208a were statistically significantly different between patients with the CC genotype compared to those with AA and AC genotypes. For all of these miRNAs, expression in patients with the CC genotype were decreased (Wilcoxon rank-sum test - miR-155, $p = 0.009$; miR-654, $p = 0.02$; miR-663, $p = 0.03$; miR-133a, $p = 0.04$; miR-208a, $p = 0.049$). There was no statistically significant difference in miRNA expression between patients who had ICD therapies and those who did not. However, consistent with expression in patients with the CC genotype, miR-155 levels were lower in patients who experienced ICD therapy and displayed the lowest p value among the 13 miRNAs on the rank-sum test ($p = 0.09$). Furthermore, when the odds ratio for having an ICD event was assessed for subjects below the 25th percentile compared with those above the 25th percentile of levels of each miRNA, only miR-155 had a statistically significantly elevated odds ratio (6.9, $p = 0.015$, Fisher's exact test). These data indicate that the CC genotype is associated with decreased expression of several miRNAs, but only the level of miR-155 is associated with ICD events.

DISCUSSION

The identification of an association between lethal arrhythmias and common genetic variants may have an important role in assisting in primary prevention as well as improving utilization of life-saving therapies. Our study shows that the AT1-1166 CC genotype conferred increased risk of ventricular arrhythmias in a chronic HF cohort. We also demonstrate that, among several miRNAs whose circulating levels were lower in HF patients with the CC genotype compared to those with one or two A alleles, miR-155 had the statistically strongest association with ICD events. These data indicate that altered regulation of miR-155 in patients with the CC genotype was predictive of increased risk of ventricular arrhythmias.

RAAS plays an important role in the regulation of physiological and pathophysiological responses such as arterial vasoconstriction, blood pressure, aldosterone production, cardiac fibrosis, cardiac hypertrophy and renal function. Most of the physiological effects of angiotensin II (Ang II) are mediated by AT1R (31, 32), which activates several intracellular signaling pathways in vascular smooth muscle cells, cardiomyocytes, and the cardiac conduction system (33). We focused on two genetic polymorphisms that are known to induce functional changes in the RAAS because there is evidence that pharmacologic

blockade of RAAS decreases SCD in patients with cardiovascular disease (34–36). Furthermore, Anvari et al identified an association between the ACE I/D and AT1-1166 CC polymorphisms and malignant ventricular arrhythmias in a cohort of patients with coronary artery disease and left ventricular dysfunction (37).

Recently, a molecular mechanism by which activation of the RAAS induces arrhythmias was described in a study of an animal model for sudden cardiac death (38). In this study, angiotensin II (acting through the AT1 receptor) induced myocyte uncoupling and sudden arrhythmic death by decreasing the myocyte gap junction protein connexin-43. Interestingly, the incidence of ventricular arrhythmias in these animals was reduced by inhibition of the tyrosine kinase c-Src, which also restored connexin-43 levels.

Similar to other reports (39), we did not find an association between ACE I/D polymorphism and ICD therapies. We could not assess a protective effect of ACE inhibitors, ARBs and β -blockers on arrhythmic risk because the majority of our patients were appropriately treated with these medications. Nonetheless, carrying the AT1-1166 CC carrier state conferred increased risk for ventricular arrhythmias independent of treatment with ACE inhibitors or ARBs.

The AT1 receptor A1166C single nucleotide polymorphism (SNP) (rs5186) is located in the 3' untranslated region (UTR) on the q22 band of chromosome 3. Previous studies have shown that the AT1-1166 CC polymorphism is associated with endothelial dysfunction, early development of hypertension, coronary atherosclerosis, MI, and left ventricular dysfunction (40, 41). This is believed to be related to changes in the density of AT1 receptors in target tissues, resulting in increased sensitivity to AT II (42–44) and subsequent activation of heterotrimeric G proteins and production of second messengers, such as inositol trisphosphate, diacylglycerol and reactive oxygen species (45). Recently, it was shown that AT1R expression could be regulated by miR-155 through binding to the AT1R mRNA 3'UTR, at the site of the AT1-A1166C SNP (17). Importantly, in the presence of the A allele, miR-155 is able to bind to the AT1R mRNA and suppress AT1R expression. In contrast, the C allele interrupts the base pairing complementarity of miR-155 with the AT1R mRNA sequence, and miR-155 is considerably less effective at suppressing AT1R expression (17, 18, 46). Thus, altered regulation by miR-155 is one mechanism by which the AT1-1166 CC polymorphism can lead to increased expression of the AT1R and cardiovascular disease.

MiRNAs have been found in whole blood, serum, plasma and other body fluids in a stable form that is protected from endogenous RNase activity (8, 47). Although the biological function of circulating miRNA is yet to be fully understood, levels of specific miRNAs (42) have been shown to correlate with pathological development of different diseases (22), and circulating miRNAs have emerged as biomarkers for disease activity and response to therapy. Importantly, circulating miRNAs can be taken up by various tissues and subsequently modulate gene expression (48). We assessed miRNA expression in whole blood, so it is possible that the levels of miRNAs that we observed were reflective of levels in circulating cells such as lymphocytes or monocytes. Indeed, Ceolotto et al. recently reported that miR-155 levels were decreased in peripheral blood mononuclear cells of hypertensive patients with the AT1-1166 CC genotype (42).

We can only speculate why levels of miR-155, miR-654, miR-663, miR-133a, and miR-208a were decreased in patients with the 1166C allele, but it is possible that these miRNAs in blood were being removed from the circulation as a means to compensate for the disease process, and, in the case of miR-155, ineffective targeting of AT1R mRNA in tissues. This mechanism for miR-155 would suggest that AT1R levels in the tissues

influence circulating miRNA levels, with higher AT1R expression resulting in lower circulating miR-155 levels. Alternatively, the CC genotype may affect miRNA transcription, processing, or stability. Notably, the expression of Dicer, one of the key ribonucleases involved in miRNA processing and synthesis was attenuated in patients with advanced HF (49). It remains to be determined whether down regulation of miRNAs in patients with the 1166C allele is directly involved in the disease process or as a compensatory response. Changes in miR-155 expression observed in our study will need to be replicated in larger studies as a necessary step to validate its candidacy as a biomarker or potential therapeutic target.

In summary, the risk for malignant tachyarrhythmias in patients with chronic HF is significantly associated with AT1-1166 CC genotype. This genotype is also associated with decreased levels of circulating miRNAs, particularly miR-155. Our study provides novel observations regarding genetic predisposition to ventricular arrhythmias in patients with chronic HF that links AT1R-1166 A/C alleles with circulating miR-155 levels and arrhythmic risk. These findings will need validation in larger studies of well-phenotyped cohorts, and functional genomic approaches are needed to elucidate the mechanistic link of circulating miR-155 levels and AT1 receptor genotype on arrhythmic risk.

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Figure 1. Determination of ACE genotypes by PCR amplification

Representative agarose gel (1%, stained with ethidium bromide) containing PCR products. Lane 1 - negative control (NC); lanes 2, 5 and 6 - ID genotypes; lane 3 - II genotype; and lane 4 - DD genotype. This analysis was performed on the 485 subjects in the study.

Figure 2.

Levels of miR-155 in whole blood of patients with 1166AA (n=25), 1166AC (n=21), and 1166CC (n=6) genotypes. Data shows the mean $\pm$ SEM for qRT-PCR analysis of miR-155 (copy numbers) in RNA extracted from whole blood samples (PAXgene Blood RNA Tubes). Ct values were converted into copy numbers ($\text{copy\#} = 2^{(-\text{Ct})}$) and normalized to RNU48. *p = 0.006, versus AA, Wilcoxon rank-sum test. A linear trend test across the 3 genotypes of the mean of the logarithm of miR-155 yields a p value of 0.002.

Table 1

Clinical and demographic characteristics of total cohort categorized by ICD therapy

	No ICD Therapy (n=299)	ICD Therapy (n=186)	p-Value
Age ¹	61 ±13	59 ±14	0.10
Gender (male)	230 (77%)	154 (82.8%)	0.12
Race ²			
Caucasian	211 (71%)	115 (62%)	0.02
African American	78 (26%)	67 (36%)	
Cardiomyopathy			
Ischemic	190 (64%)	124 (67%)	>0.20
Nonischemic	82 (27%)	43 (23%)	
Other	26 (9%)	19 (10%)	
LVEF ¹	21±6.5	19±7	0.01
NYHA Class ¹	2.0±0.7	2.1±0.7	0.07
Diabetes	113 (38%)	75 (40%)	>0.20
Hypertension	203 (68%)	127 (68%)	>0.20
Dyslipidemia	194 (63%)	123 (66%)	>0.20
Smoking	168 (55%)	119 (64%)	0.10
ACE Inhibitor	192 (64%)	109 (59%)	>0.20
ARB	36 (12%)	28 (15%)	>0.20
β-Blocker	257 (86%)	161 (87%)	>0.20

¹Values are presented as mean ± SD²The missing counts are subjects of race other than Caucasian or African American.

LVEF: Left Ventricular Ejection Fraction; NYHA: New York Heart Association; ACE: Angiotensin Converting Enzyme; ARB: Angiotensin Receptor Blocker.

Table 2

Distribution of the ACE I/D and AT1R-1166 genotypes by ICD Therapy with Odds Ratios and 95% Confidence Intervals for Caucasians and African Americans

ACE	Caucasians		African Americans		OR ¹	95% CI
	No Therapy	ICD Therapy	No Therapy	ICD Therapy		
II	27 (13%)	18 (16%)	11 (14%)	11 (16%)	1.0	
ID	95 (45%)	52 (46%)	39 (50%)	25 (37%)	0.83	0.48, 1.5
DD	89 (42%)	43 (38%)	28 (36%)	31 (46%)	0.76	0.44, 1.3
$\chi^2 = 0.14$ P>0.20 ²						
AT1R						
AA	97 (46%)	46 (40%)	62 (79%)	57 (85%)	1.0	
AC	101 (48%)	54 (47%)	16 (21%)	8 (12%)	1.0	(0.66, 1.5)
CC	13 (6%)	15 (13%)	0 (0%)	2 (3%)	2.5	(1.1, 5.5)
$\chi^2 = 2.25$ P=0.13 ²						

¹Odds of therapy for this genotype divided by the odds for base category adjusted for race.

²Trend Chi-squared test for association adjusted for race.

³Odds ratio and chi-squared tests include 14 subjects (10 no therapy, 4 therapy) of other race.

⁴Odds ratio for CC versus (AA or AC) = 2.52 (1.2, 5.4)95% P = 0.02.