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A Novel Genetic Marker of Decreased Inflammation and Improved Survival after Acute Myocardial Infarction

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Abstract

Background: The *CHRNA5* gene encodes a neurotransmitter receptor subunit involved in multiple processes, including cholinergic autonomic nerve activity and inflammation. Common variants in *CHRNA5* have been linked with atherosclerotic cardiovascular disease. Association of

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variation in *CHRNA5* and specific haplotypes with cardiovascular outcomes has not been described.

Objective: To examine the association of *CHRNA5* haplotypes with gene expression and mortality among patients with acute myocardial infarction (AMI) and explore potential mechanisms of this association.

Methods and Results: Patients (N=2054) hospitalized with AMI were genotyped for two common variants in *CHRNA5*. Proportional hazards models were used to estimate independent association of *CHRNA5* haplotype with 1-year mortality. Both individual variants were associated with mortality ($p = 0.0096$ and 0.0004 , respectively) and were in tight LD ($D' = 0.99$). One haplotype, HAP3, was associated with decreased mortality one year after AMI (adjusted HR = 0.42 , 95% CI $0.26, 0.68$; $p = 0.0004$). This association was validated in an independent cohort (N=637) of post-MI patients (adjusted HR= 0.23 , 95% CI $0.07, 0.79$; $p = 0.019$). Differences in *CHRNA5* expression by haplotype were investigated in human heart samples ($n=28$). Compared with non-carriers, HAP3 carriers had threefold lower cardiac *CHRNA5* mRNA expression ($p=0.023$). Activation of the inflammasome, an important inflammatory complex involved in cardiovascular disease that is necessary for release of the pro-inflammatory cytokine IL-1 β , was assessed in bone marrow derived macrophages (BMDM) from *CHRNA5* knockout mice and wild-type controls. In BMDM from *CHRNA5* knockout mice, IL-1 β secretion was reduced by 50% compared to wild-type controls ($p= 0.004$).

Conclusion: A common haplotype of *CHRNA5* that results in reduced cardiac expression of *CHRNA5* and attenuated macrophage inflammasome activation is associated with lower mortality after AMI. These results implicate *CHRNA5* and the cholinergic anti-inflammatory pathway in survival following AMI.

Keywords

cardiovascular disease; genetic variation; myocardial infarction; mortality; haplotype; inflammation

Introduction

Atherosclerotic cardiovascular disease is the most common cause of death in the United States.[15] Coronary artery disease (CAD) and acute myocardial infarction (AMI) are polygenic diseases with high heritability.[36] Genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) associated with the risk of CAD, AMI, and coronary artery calcification,[5] but the association of genetic variation with outcome following AMI, and the potential biologic mechanism(s) mediating the effects of genetic variants on outcomes have been less well investigated.

In animal models of myocardial ischemia/reperfusion, vagal stimulation results in decreased infarct size,[6] a decreased number of infiltrating macrophages and polymorphonuclear leukocytes, and decreased circulating cytokines, and these effects occur independent of heart rate.[6] Vagal inhibition of systemic inflammation is mediated through nicotine acetylcholine receptors (nAChRs).[40, 42] nAChRs are ligand-gated heteropentameric ion channels present in neuronal and non-neuronal cells, and are activated by both nicotine and

acetylcholine. Of note, the alpha5 nAChR and the alpha7 nAChR are the only two subunits with both high mRNA and protein expression in cardiac tissue.[38] The alpha7 receptor is predominantly homopentameric and is a key regulator in vagal-mediated anti-inflammatory pathways.[40, 42] The alpha5 receptor forms heteropentamers with other nAChR subunits, including alpha7, and inhibition or antagonism of alpha5 nAChR pathways appears to up-regulate nAChR alpha7 pathways.[3]

Variants in *CHRNA5*, the gene that encodes the alpha5 nAChR, have shown association with tobacco dependence [4, 7] and the risk of chronic diseases, such as lung cancer,[50] chronic obstructive pulmonary disease,[50] diabetes,[48] and atherosclerotic peripheral vascular disease (PVD).[49] Notably, the associations of *CHRNA5* with diabetes and PVD were found to be independent of smoking status.[49] The functional consequences of *CHRNA5* variants and their haplotypes on cardiac gene expression and their potential association with post-MI inflammation and cardiovascular clinical outcomes have not been investigated.

We therefore sought to investigate the association of *CHRNA5* variants, individually and as haplotypes, with subsequent mortality among patients following AMI. To explore putative mechanisms for observed associations, we assessed the functional consequences of *CHRNA5* variants and their haplotypes on gene expression in cardiac tissue from an independent set of myocardial samples stratified by haplotype. To explore the potential contribution of inflammation as a mechanism for this association, we assessed the response of bone marrow derived macrophages (BMDM) harvested from *CHRNA5* knockout mice, compared with wild type littermates, to inflammatory stimulation.

METHODS

TRIUMPH Cohort

We examined the association between variation in *CHRNA5* and outcomes in the large, multicenter Translational Research Investigating Underlying disparities in acute Mycocardial infarction Patients' Health status (TRIUMPH) cohort of patients prospectively enrolled after hospitalization for AMI.[2] Between April 11, 2005, and December 31, 2008, 4340 patients with AMI, from 24 U.S. hospitals were prospectively enrolled into the TRIUMPH observational cohort study, as previously described.[2, 10, 19] AMI patients were identified by an elevated troponin blood test and either diagnostic electrocardiogram (EKG) changes or ischemic symptoms. 2979 TRIUMPH patients consented to genetic testing.[19] Of these, 2955 (99.2%) were discharged alive and were included in the present analyses. Given the large frequency differences for several genotypes of interest across race, and, in particular, the low frequency of the *CHRNA5*rs16969968 variant in African Americans,[4] we restricted the analyses to self-identified Caucasians. Subjects reporting both Caucasian and African American race (N=27) were excluded from the analysis. The final discovery cohort consisted of 2062 TRIUMPH post-MI patients. The representativeness of the TRIUMPH genetics cohort (compared to the entire TRIUMPH cohort) has previously been reported.[19]

Each patient was prospectively interviewed during their AMI hospitalization to ascertain their socio-demographic (including self-identified race), economic and health status characteristics. Detailed chart abstractions were performed to obtain patients' medical

history, laboratory results, disease severity and the processes of inpatient care. TRIUMPH received Institutional Review Board approval at all participating sites and written informed consent was obtained from each participant.

Validation Cohort

Our validation cohort was derived from two consecutive multicenter, prospective cohort studies of patients hospitalized with AMI in the U.S.: 1) the Investigations of Outcomes From Acute Coronary Syndromes (INFORM) study enrolled 1199 patients with acute coronary syndrome (680 AMI; 519 unstable angina) from 2 US hospitals between March 2001 and October 2002, and 2) the Prospective Registry Evaluating Myocardial Infarction: Events and Recovery (PREMIER) study enrolled 2498 patients with AMI from 19 US hospitals between January 2003 and June 2004.[9] Both studies were coordinated by Saint Luke's Mid America Heart Institute and employed identical inclusion and exclusion criteria for AMI (for validation, only AMI INFORM subjects were included). A total of 1154 genetic samples were available for analysis from INFORM/PREMIER (PREMIER obtained genetic samples from only 2 of the 19 sites). Of these, 853 were from Caucasian subjects; 212 subjects from INFORM with a diagnosis of unstable angina and not AMI were excluded. The final validation cohort, therefore, was comprised of 641 post-MI patients.

Outcome Assessment

One-year mortality was the primary outcome and the outcome used for validation. The Social Security Administration Death Master File was queried to determine patients' vital status as of 12/31/2010 (<http://www.ntis.gov/products/ssa-dmf.asp>) and was available for all patients in this study. Of note, this query was performed prior to new restrictions and expunging of some records from the database. To assess potential contributors to the lower mortality observed among HAP3 carriers, the outcomes of recurrent AMI, cardiac rehospitalization, and non-elective cardiac rehospitalization (limiting cardiac rehospitalization to non-elective admission only) were also determined in TRIUMPH for HAP3 carriers and non-carriers as an exploratory analysis. TRIUMPH follow-up and outcome ascertainment and adjudication have previously been described.[2, 9, 12]

Genotyping

DNA was isolated and purified from whole blood using the Qiagen QIAamp DNA purification kit (Quiagen, Germantown, MD). Genotyping was performed using the Infinium HumanCore BeadChip (Illumina, CA, USA) and HumanExome Array V1.2(Illumina, CA, USA) as per manufacturer's instructions. Call rate was 98.6% for rs680244 and 97.4% for rs16969968. Some samples had also been previously genotyped using pyrosequencing assay[7] (i.e. we had duplicate data for some of the same samples); duplicate data had correlation coefficient = 0.979.

mRNA Expression

Cardiac tissue samples were collected from 28 hearts either donated for orthotopic heart transplant but declined for reasons related to size or ABO mismatch (n=21), or explanted from transplant recipients (n=7). Consent for donation of cardiac tissue for research

purposes was obtained from family members by the organ donor organization covering the Colorado–Wyoming–Montana region (Donor Alliance). DNA was extracted from donor whole blood samples, genotyped and haplotype imputed as described below. Total RNA isolation from cardiac tissue utilized an RNeasy® kit (Qiagen Inc., CA, USA) with the addition of DNase and reverse transcribed into cDNA using the SuperScript III™ first strand cDNA synthesis kit (Invitrogen, CA, USA) and random primers. Gene expression was detected using TaqMan (Applied Biosystems, CA, USA) assays of *CHRNA5* (Hs00181248_m1), and *GAPDH* (Hs99999905_m1). Reactions were performed in triplicate using the ABI7300 system. Comparative Ct method was used to quantify mRNA expression levels of target genes with data normalized to *GAPDH* expression.[21] Expression of *CHRNA5* was compared between declined (n=21) and explanted (n=7) specimens. As no significant difference was observed in *CHRNA5* (p=0.647) mRNA expression, these specimens were combined for the primary analysis of mRNA expression between HAP3 carriers and non-carriers. A sensitivity analysis was also performed using declined specimens only.

Macrophage Experiments

Long bones from *CHRNA5* knockout mice (backcrossed to C57BL/6J for more than 15 generations) and C57BL/6J controls were shipped overnight on ice (from Stitzel laboratory to Cresci laboratory). Bone marrow-derived macrophages (BMDM) were prepared by harvesting bone marrow from the femurs and tibias of 15–16-week-old mice as previously described.[46] The cells were seeded in 10 cm dishes and differentiated for 6 days in DMEM media (containing 10% inactivated fetal serum, 50 U/mL penicillin G sodium, and 50 U/mL streptomycin sulfate (pen-strep), 2 mM L-glutamine, and sodium pyruvate) supplemented with 10% supernatant from CMG14.12 cells as a source of M-CSF.[33] On day 6, BMDMs were plated at 1×10^6 cells/mL in media containing 5% supernatant from CMG14.12 cells. Stimulations were performed on the day after plating in media containing 5% CMG1.12 media. Cells were removed from the plate by washing with PBS followed by 10 minutes with Cell Stripper (GIBCO) and then 10 minutes with EDTA/trypsin (Sigma). To measure NLRP3 inflammasome activation, a two-signal stimulation was performed as previously described.[33, 46] LPS (50 ng/mL; priming signal) was added to cells for 20h followed by vehicle or ATP (4mM; signal 2) for 30 minutes. Supernatants were harvested from macrophage cultures after the indicated stimulations. IL-1 β was quantified using a DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions.

All human and animal studies have been approved by the appropriate institutional review boards and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Statistical Analyses

Our primary aim was to identify the association of two polymorphisms (rs680244 and rs16969968) within the *CHRNA5* region, individually and in haplotype analysis, with 1-year mortality among Caucasian post-MI TRIUMPH patients after discharge from the index AMI hospitalization. Individual association analysis was performed using Cox proportional hazard models.

For haplotype analyses, the *CHRNA5* region was first explored through Haploview to examine linkage disequilibrium (LD), Hardy Weinberg Equilibrium (HWE) of individual SNPs, and haplotype frequency. Using THESIAS[37] stochastic EM algorithm (i.e. treating each haplotype as an independent entity and not considering haplogenotype) for Cox proportional hazards survival regression, we tested the association with mortality of the three haplotypes. Haplotype phasing was finalized using PHASE,[31] where imputation quality could be output for sensitivity analysis. Haplotypes with imputation quality confidence >0.6 were considered in sensitivity analyses. Haplotype phase could not be determined for eight TRIUMPH patients and four INFORM/PREMIER patients and these subjects were, therefore, excluded from analysis, resulting in a final TRIUMPH cohort of 2,054 subjects and a final INFORM/PREMIER cohort of 637 subjects.

Subsequent analyses of collapsed haplotype groups were performed in SAS version 9.2 (SAS Institute, Inc., Cary, NC) and R version 2.11.1, where a stratified and unadjusted model was used for initial log rank (Kaplan Meier survival) analyses. Baseline and follow-up characteristics were compared as follows: categorical data are reported as frequencies, with differences between groups compared using chi-square or Fisher's exact tests, as appropriate. Continuous data are reported as mean $\pm$ standard deviation (SD) with differences between groups tested using independent sample t-tests. To estimate the independent contribution of haplotype, stratified proportional hazards models were adjusted for sex, GRACE score,[47] and any covariate differing between carriers and non-carriers of the protective haplotype for all outcomes. Hazard ratios were determined for carrier status of the protective haplotype compared to carriers of all other haplotypes. Validation analyses were performed similar to the primary analyses.

As we, and others, have observed an association of *CHRNA5* rs16969968 with smoking cessation,[7, 8] haplotype by smoking (defined at baseline as active, former (e.g. >1 month prior to baseline), or never) interaction was explored. We first forced smoking status into our adjusted models. We then formally tested for an interaction between HAP3 and smoking status with 1-year mortality. We also used stepwise selection of covariates from among GRACE score,[47] sex, smoking status, history of PVD, and the interaction between HAP3 carrier status and smoking status (with all terms allowed to enter the model, but remaining in the model contingent on a p-value of 0.10), and determined which covariates were significantly associated with mortality. Model goodness of fit was compared using Akaike information criterion (AIC).

For mRNA expression studies, haplotype phasing for cardiac tissue could be determined for all samples. Experiments were performed in triplicate. mRNA expression level values were summarized by mean and standard deviation and compared using *t*-tests.

For all analyses, p-values <0.05 were considered statistically significant; interaction p-values <0.2 were considered statistically significant.[14, 32] In TRIUMPH, given a mortality of 4.9% in 2054 Caucasians, there was 80% or more power to detect a HR of 0.59 or less (or HR 1.57) with a haplotype frequency of 0.21 or greater.

RESULTS

TRIUMPH Cohort

Between April 11, 2005, and December 31, 2008, 2062 Caucasian subjects enrolled in TRIUMPH and provided DNA samples and consent for genotyping. Haplotype phase could not be determined for eight Caucasian patients; these subjects were excluded from analysis. The final cohort, therefore, consisted of 2054 Caucasian subjects. Baseline characteristics of these subjects are shown in Table 1. The majority of subjects were male, 26.1% had diabetes, 37.2% were active smokers on admission, and 35.5% had smoked cigarettes at some time in the past. More than 80% of TRIUMPH subjects received in-hospital revascularization; the majority underwent percutaneous intervention. In these 2054 Caucasian TRIUMPH subjects, there were 101 deaths (4.9%) at one year.

Individual SNP Associations

The individual *CHRNA5* variants were common and did not deviate significantly from HWE (Supplemental Table S1). We determined the association with 1-year mortality for both of the individual variants. Each SNP demonstrated an association with mortality (unadjusted Cox proportional HR p values 0.0096 and 0.0004; p-values and HRs in Supplemental Table S2), however these variants were in tight linkage disequilibrium (LD; $D' = 0.99$, $r^2 = 0.32$; Supplemental Figure S1A) and we therefore performed haplotype analysis in order to more fully investigate the genetic contribution to mortality.

Haplotype Analysis

Three *CHRNA5* haplotypes represented 100% of the variation (Supplemental Figure S1B). The most common haplotype (designated HAP1; TG: *CHRNA5* rs680244 (T) allele, *CHRNA5* rs16969968 (G) allele, was present in 41% of the population, while the third most common *CHRNA5* haplotype (HAP3, *CHRNA5* rs680244 (C) allele, *CHRNA5* rs16969968 (G) allele) was present in 27% of the population. THESIAS analysis identified *CHRNA5* HAP3 to be significantly associated ($p = 0.0007$) with mortality at one year following AMI. We then formally assessed (i.e. taking haplogenotype into account) whether HAP3 carriers had significantly different mortality compared to subjects with HAP1 or HAP2. When compared with carriers of all other haplotypes combined (collapsed group) in SAS, HAP3 haplotype was significantly associated with lower mortality using either an additive (i.e. carriers of 2 copies vs. carriers of 1 copy vs. carriers of 0 copies of HAP3) model (log rank $p = 0.00160$; Figure 1 left) or a dominant (HAP3 carriers compared to non-carriers) model (log rank $p = 0.00041$; Figure 1 right). Using a dominant model, the HAP 3 (GC carrier) vs. non-carrier HR was 0.46 (95% CI 0.30, 0.72; $p = 0.00057$; Table 4), indicating a strong and highly significant association between HAP3 and mortality.

Baseline characteristics of TRIUMPH carriers of HAP3, as compared with carriers of any other haplotype, are shown in Table 2. No difference in resting heart rate (80.60 ± 20 versus 81.61 ± 22.8 ; $p = 0.286$) or in LDL (104.67 ± 42.4 versus 102.42 ± 38.5 ; $p = 0.235$) was observed between HAP3 carriers compared to non-carriers at admission. There were minor, but statistically significant, differences in the GRACE risk score[47] and history of PVD in HAP3 carriers compared to non-carriers (98.02 ± 29.1 versus 101.23 ± 29.8 ; $p = 0.014$ and

3.3% vs. 5.3%; $p = 0.025$, respectively). As seen in Table 3, HAP3 remained significantly associated with decreased 1-year mortality after adjusting for GRACE risk score, history of PVD, and the forced covariate, sex (HR= 0.52; 95% CI 0.34, 0.81; $p=0.00391$).

Validation

We confirmed the association of HAP3 with decreased 1-year mortality in an independent (INFORM/PREMIER) cohort of post-MI patients (N=637). As observed in the TRIUMPH population, HAP3 carrier status was significantly and strongly associated with decreased 1-year mortality (log rank $P = 0.0021$; unadjusted HR=0.19; 95% CI 0.06, 0.62; $p=6.04E-03$; Figure 2 and Table 4, respectively). Similar to TRIUMPH, after adjustment for sex and GRACE score, HAP3 remained significantly associated with decreased 1-year mortality in the validation cohort (HR=0.20; 95% CI 0.06,0.67; $p=9.13E-03$; Table 4).

Effect of Smoking

As we previously observed a significant association between *CHRNA5*rs16969968 and ability to quit smoking in the post-MI TRIUMPH cohort,[7] we explored whether smoking status affected the observed association of HAP3 with reduced mortality. First, we tested for a formal interaction between HAP3 and smoking status with 1-year mortality in TRIUMPH, and found no significant interaction (interaction $p= 0.8902$). Next, using stepwise selection of covariates from among GRACE score,[47] sex, smoking status, history of PVD and the interaction between HAP3 carrier status and smoking status, only GRACE score, history of PVD and HAP3 carrier status were significantly associated with 1-year mortality in TRIUMPH. Forcing any other term, including smoking status, into the model resulted in higher model AICs, indicating poorer model fit. Furthermore, forcing smoking status into our adjusted models did not affect the association of HAP3 carrier status with lower mortality in TRIUMPH or in the validation cohort (Tables 4 and 5). Finally, we determined the direction of HAP3 effect and found that it was similar in all groups (never smokers, former smokers and active smokers). Because the direction of HAP3 effect in never smokers in both TRIUMPH and the validation cohort was similar (with borderline significance in each cohort (log rank $p= 0.0564$ in TRIUMPH; log rank $p= 0.0845$ in the validation cohort; Supplemental Figure S2A and S2B, respectively), we combined the groups to increase power. As seen in Figure 3, in the combined group of never smokers, HAP3 carriers had significantly decreased mortality 1-year after admission for AMI (log rank p value for combined group= 0.0116; HR= 0.39; 95% CI 0.19, 0.83; $p=0.0149$). The association of HAP3 with decreased 1-year mortality in never smokers remained significant after adjusting for GRACE risk score and sex (HR= 0.39; 95% CI 0.18, 0.82; $p=0.0131$). These observations indicate that smoking status does not explain the association between HAP3 and mortality following AMI.

Exploratory Mechanisms of HAP3 Influence on Decreased Mortality

We assessed clinical factors known to have an influence on outcomes after AMI, such as age, heart rate, systolic blood pressure, presence of diabetes and kidney disease [23, 47] but there were no observed differences in these clinical factors between HAP3 carriers and non-carriers (Table 2).

Further analyses were performed in the TRIUMPH cohort in an attempt to define whether the decreased mortality observed in HAP3 carriers was related to decreased rates of recurrent cardiovascular events. When HAP3 carriers versus non-carriers were compared, there was no significant difference in recurrent myocardial infarction ($p = 0.4142$), cardiac rehospitalization ($p = 0.1875$) or non-elective cardiac rehospitalization ($p = 0.1598$) one year after admission for AMI.

To determine if the influence of HAP3 on mortality might be related to gene expression, mRNA expression of *CHRNA5* in cardiac tissue ($n=28$) was compared between HAP3 carriers and non-carriers. Carriers for HAP3 ($n=9$) had a nearly three-fold decrease in expression of *CHRNA5* ($2^{-Ct} = 2^{-1.50}$, $p=0.009$; Figure 4A) compared with non-carriers ($n=19$). We hypothesized that the decreased expression of *CHRNA5* in HAP3 carriers would lead to decreased inflammation mediated by the cholinergic anti-inflammatory pathway. We therefore assessed whether there was a difference in the circulating inflammatory marker high-sensitivity C-reactive protein (hsCRP) in TRIUMPH HAP3 carriers versus non-carriers and found that circulating levels of hsCRP were significantly lower in HAP3 carriers versus non-carriers (3.43 ± 4.2 versus 3.91 ± 5.1 ; $p=0.0379$; Figure 4B). The NLRP3 inflammasome is a potent inflammatory complex and activation of this complex is thought to contribute to the development of unstable atherosclerotic plaques[20] and post-MI LV dysfunction.[24] To test the hypothesis that decreased expression of *CHRNA5* would suppress the NLRP3 inflammasome, BMDM were harvested from *CHRNA5* knockout mice or wild-type controls, primed with LPS followed by ATP stimulation, and IL-1 β production measured, as previously described.[33, 46] As seen in Figure 5, after stimulation, BMDM harvested from *CHRNA5* knockout mice released 50% less IL-1 β compared to BMDM from wild-type mice ($p=0.004$).

DISCUSSION

We have identified a common *CHRNA5* haplotype that is associated with altered cardiac gene expression and significantly lower mortality 1 year following AMI. Although prior investigations had observed associations between individual SNPs within this region and select manifestations of atherosclerotic vascular disease such as the presence of PVD,[44, 49] no previous studies have demonstrated a relationship of this gene with clinical outcomes among patients following AMI. We observed that carriers of one haplotype, HAP3, found in 27% of Caucasian TRIUMPH subjects, have less than half of the 1-year mortality after hospitalization for AMI when compared with non-carriers. Importantly, the highly significant association of HAP3 with mortality following AMI is independent of potential confounders such as comorbidity and smoking status, and was validated in an independent cohort of post-MI patients, supporting the influence of this chromosomal region on post-MI clinical outcomes. Our analyses correlating haplotype with gene expression suggests a potential mechanism of this association that is further supported by levels of circulating inflammatory markers in TRIUMPH and our data from BMDM harvested from *CHRNA5* knockout mouse. Collectively, our data supports that the lower 1-year mortality observed in *CHRNA5* HAP3 carriers may be related to differences in the inflammasome-mediated response to AMI.

To date, limited studies have investigated associations between genetic variants and clinical events after AMI. Moss and colleagues studied the association of 18 independent genetic variants that had been previously identified as predictive of higher risk of CAD among 1008 patients who had survived a recent AMI.[26] They observed that only a single variant in the angiotensin receptor gene (rs5186) showed a significantly lower risk (HR 0.75, 95% CI 0.58–0.97) of the combined endpoint of recurrent AMI, unstable angina and death from CAD.[26] We previously examined 95 polymorphisms in 69 distinct gene regions identified in GWAS for premature AMI for their association with mortality among 811 patients following an acute coronary syndrome, 73% of whom had AMI.[25] We observed that, after adjustment for other risk factors, of the 95 SNPs tested only the rs6922269 polymorphism in the methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like gene (*MTHFD1L*) showed a statistically significant association with increased mortality, but even that association was not confirmed in an independent cohort.[25] Wauters and colleagues investigated whether 23 loci previously identified by GWAS studies as associated with CAD would predispose 2099 patients with an acute coronary syndrome participating in the GRACE registry to recurrent AMI or cardiac death during 5 years of follow-up.[45] They observed significant associations for a variant in the ABO locus on chromosome 9 and the rs1333049 variant at the 9p21 locus with the combined outcome of recurrent AMI and cardiac death.[45] However, we, and others, have failed to confirm an association of any variant in the 9p21 chromosome region with death, recurrent AMI, or revascularization following AMI.[13, 17, 25, 41] More recently, Andreasse and colleagues genotyped 498 patients with a diagnosis of ischemic heart disease (63% had a history of AMI) for 48 SNPs previously reported to be independently associated with CAD or AMI and examined their association with composite major adverse cardiac events (MACE) including coronary-related death, nonfatal AMI or revascularization over a mean of 6.9 years.[1] When 5 previously identified high-risk SNPs were combined into a risk score, the summed genetic score predicted a significant 25% increase risk of MACE per risk allele. Of note, among the published analyses reporting genetic associations with outcome for patients with ischemic heart disease, the current report is the first to identify that genetic variation in the region of 15q25 is associated with mortality after AMI.

The *CHRNA5* haplotype identified in our analyses as having a significant association with post-MI mortality, HAP3, is composed of 2 SNPs, *CHRNA5* rs680244 (C) allele and *CHRNA5* rs16969968 (G) allele. Previous investigators observed that the *CHRNA5* rs16969968 SNP, and *CHRNA5* SNPs in tight LD with rs16969968, were associated with increased PVD in European and American Indian cohorts and that this association was independent of smoking status.[34, 49] No previous studies have shown associations of these variants with cardiovascular clinical outcomes. The two *CHRNA5* SNPs in HAP3 have been shown *in vitro* to result in functional changes. The *CHRNA5* rs16969968 variant results in an amino acid change (D398N) in the alpha5 subunit of nAChR that leads to an altered receptor response to agonist; the receptor containing asparagine at residue 398 (N398) exhibits a lower maximal response to nicotinic agonist compared with the receptor containing aspartic acid at residue 398 (D398).[4] The *CHRNA5* rs680244 variant is a C to T nucleotide change that has been shown to alter mRNA expression in human brain tissue samples.[30, 43]

Since the mechanism(s) by which these functional changes in gene expression or protein activity due to HAP3 may affect risk of mortality among patients following AMI are unknown, we performed additional analyses to investigate potential clinical mediators of variation in outcome. Of note, we did not observe significant differences in recurrent adverse ischemic events including myocardial infarction ($p = 0.942$), cardiac rehospitalization ($p = 0.864$) or non-elective cardiac rehospitalization ($p = 0.950$) at one year after hospitalization for AMI among HAP3 carriers versus non-carriers. These observations suggest a mechanism for HAP3 effect that is independent from, or additive to, the clinical consequences of atherosclerosis.

Our experiments examining *CHRNA5* gene expression in cardiac tissue also support a mechanism for HAP3 effect that is distinct from atherosclerosis. These data demonstrate that cardiac *CHRNA5* mRNA expression is decreased in HAP3 carriers compared with non-carriers. *CHRNA5* codes for the alpha5 subunit of nAChR and inhibition or antagonism of alpha5 nAChR pathways appears to up-regulate alpha7 nAChR pathways.[3] Proposed mechanisms include that the alpha5 subunit is a component of other nAChRs that are negative regulators of the alpha7 pathway and/or that alpha5 subunits competitively complex directly with alpha7 subunits, thereby decreasing or in some way, altering their function.[3]

There is a complex interplay between vagal activation of the alpha7 nAChR pathway and the intrinsic cardiac nervous system (comprised of intrinsic cardiac ganglia that can influence cardiac function independent of CNS influence).[16, 28, 29, 39, 51] Vagal activation of the alpha7 nAChR pathway is known to suppress the production of pro-inflammatory cytokines such as TNF, IL-1 β , and IL-6 by macrophages[40, 42, 51] and to decrease infarct size in animal models of AMI, independent of heart rate.[6] This effect is thought to be partly mediated by circulating factors that act on specific intrinsic cardiac ganglia, leading to a release of acetylcholine in the ventricular myocardium and activation of muscarinic receptors.[29]

Since activation of macrophage-mediated inflammation via the NLRP3 inflammasome complex has been shown to contribute to post-MI inflammation,[20] we speculate that one possible mechanism for the decreased mortality observed in TRIUMPH HAP3 carriers is that decreased expression of *CHRNA5* mRNA in HAP3 carriers results in decreased levels of alpha5 nAChR subunits, increased activation of alpha7 nAChR pathways, and consequently reduced tissue inflammation and adverse consequences of ischemic injury. The fact that TRIUMPH HAP3 carriers had significantly lower hsCRP levels compared to non-carriers supports this hypothesis.

Human post-MI autopsy samples and animal models of AMI also support this hypothesis. AMI is associated with a robust infiltration of inflammatory cells consisting of monocytes, macrophages, and neutrophils.[11, 18, 24, 27] More recently, the NLRP3 inflammasome, and its product cytokine IL-1 β , have been implicated in post-MI inflammation and adverse LV remodeling.[11, 20, 24, 27] Investigators have shown that activation of the inflammasome promotes adverse cardiac remodeling and fibrosis and that inhibiting the inflammasome limits infarct size and area at risk following AMI.[18, 24, 35] Extracellular ATP is an important activator of the NLRP3 inflammasome after ischemic injury.[22]

Therefore we investigated the inflammasome response of BMDMs derived from *CHRNA5* deficient mice in response to ATP stimulation. Consistent with our hypothesis, macrophages from *CHRNA5* knockout mice released less IL-1 β . These observations support the concept that reduced expression of alpha5 nAChR subunits may have a positive influence on alpha7-mediated inhibition of the inflammasome (i.e. HAP3 carriers have increased vagal-intrinsic cardiac nervous system activation which leads to decreased inflammasome activation) that may lead to differential post-translational processing of pro-IL-1 β by the NLRP3 inflammasome and/or decreased priming leading to decreased IL-1 β release. The resultant reduced inflammatory response would be predicted to improve myocardial recovery and remodeling, and, ultimately, could contribute to the decreased post-MI mortality we observe in HAP3 carriers. The fact that TRIUMPH HAP3 carriers had significantly decreased levels of the circulating inflammatory marker hsCRP at the time of presentation to the hospital with an acute MI, supports that, regardless of the exact mechanism, these patients had less inflammatory response to AMI. However additional investigation will be required to link this decreased inflammatory response to the reduced post-MI mortality seen in HAP3 carriers and to comprehensively define the mechanistic link between decreased *CHRNA5* expression and the inflammasome response.

Our study should be interpreted in the context of several potential limitations. First, our investigation was limited to Caucasians to conform to prior association studies and because of racial frequency differences (in particular, the low frequency of the *CHRNA5* rs16969968 variant in African Americans.[4]) Second, although our findings were validated in an independent post-MI cohort and the association of HAP3 with decreased 1-year mortality was supported, we acknowledge the need for further investigations into the mechanisms of this association. Lastly, we recognize the challenge of discriminating the effect of smoking status as well as smoking cessation on this observational cohort and the potential confounding of this with HAP3. Our steps to adjust based on this potential confounder were meticulous and extensive, however, and do suggest a strong and independent effect of HAP3. Furthermore, our data of a mortality benefit in HAP3 carriers in subjects who have never smoked strongly supports that the described association is independent of smoking.

CONCLUSION

In conclusion, we have identified a common *CHRNA5* haplotype associated with significantly decreased cardiac *CHRNA5* mRNA expression, significantly decreased levels of the circulating inflammatory marker hsCRP, and significantly decreased 1-year mortality following AMI. We further demonstrated that BMDM from the *CHRNA5* knockout mouse had decreased NLRP3 inflammasome activation as measured by IL-1 β release and hypothesize that decreased inflammasome activation leads to decreased inflammation, improved myocardial recovery and remodeling, and decreased post-MI mortality in HAP3 carriers. These results, in aggregate, implicate a novel nicotinic cholinergic receptor pathway in survival following AMI, and identify both a new genetic biomarker for prediction of survival after AMI and a potential therapeutic target for personalized treatment of AMI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations List:

AMI	Acute Myocardial Infarction
BMDM	Bone Marrow Derived Macrophages
CAD	Coronary Artery Disease
GWAS	Genome-Wide Association Study
HWE	Hardy Weinberg Equilibrium
PVD	peripheral vascular disease
LD	Linkage disequilibrium
MACE	Major Adverse Cardiac Events

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Figure 2.
Kaplan-Meier Survival curves for Caucasian *CHRNA5*HAP3 (CG) carriers according to HAP3 copy number (A) and as a dominant model (carriers vs. non-carriers; B) in an independent validation cohort.

Figure 3.
Kaplan-Meier Survival curves for Caucasian *CHRNA5*HAP3 (CG) carriers never smokers.

Figure 4.

(A) Relative *CHRNA5* mRNA expression in cardiac tissue stratified by HAP3 carrier status. Error bars represent one standard deviation above and below the mean values of relative mRNA expression. (B) Circulating inflammatory marker high-sensitivity C-reactive protein (hsCRP) in TRIUMPH subjects stratified by HAP3 carrier status.

Figure 5. IL-1 β (pg/ml) by ELISA in BMDM from *CHRNA5* knockout mice (KO) versus non-transgenic controls (WT).

Table 1.

Baseline characteristics, in-hospital revascularization, and discharge medications in TRIUMPH cohort

	Caucasian (N = 2054)
Age, years	59.78 ± 12.1
Male	1482 (72.2)
BMI, kg/m ²	29.61 ± 6.2
Smoking Status	
Never Smoker	558 (27.4)
Former Smoker	724 (35.5)
Active Smoker	758 (37.2)
Hypertension	1260 (61.3)
Diabetes	536 (26.1)
Hyperlipidemia	1040 (50.6)
Chronic Kidney Disease	101 (4.9)
Hx of CHF	113 (5.5)
PVD	90 (4.4)
Prior MI	381 (18.5)
Prior PCI	412 (20.1)
Prior CABG	251 (12.2)
Prior CVA	80 (3.9)
Prior TIA	52 (2.5)
STEMI	982 (47.8)
NSTEMI	1056 (51.4)
GRACE Risk Score	99.79 ± 29.5
In-hospital revascularization	
CABG	201 (9.8)
PCI	1452 (70.7)
Discharge Medications	
Aspirin	1963 (95.6)
Beta Blocker	1881 (91.6)
ACE/ARB	1518 (73.9)
Lipid Lowering	1865 (90.8)

Values are mean ± SD or N (%).

Table 2.

Baseline characteristics, in-hospital revascularization, and discharge medications in TRIUMPH Caucasian HAP3 carriers versus non-carriers

	Carriers (N =920)	Non Carriers (N= 1134)	p-value
Age, years	59.31 (12.2)	60.15 (12.0)	0.116
Male	652 (70.9)	830 (73.2)	0.243
Heart Rate	80.60 (20)	81.61 (22.8)	0.286
BMI, kg/m ²	29.88 (6.2)	29.38 (6.2)	0.074
Smoking Status			0.544
Never Smoker	245 (26.9)	313 (27.7)	
Former Smoker	315 (34.6)	409 (36.2)	
Active Smoker	350 (38.5)	408 (36.1)	
Hypertension	566 (61.5)	694 (61.2)	0.881
Diabetes	242 (26.3)	294 (25.9)	0.846
Hyperlipidemia	466 (50.7)	574 (50.6)	0.988
LDL	104.67 (42.4)	102.42 (38.5)	0.235
Chronic Kidney Disease	42 (4.6)	59 (5.2)	0.506
Hx of CHF	48 (5.2)	65 (5.7)	0.611
Hx of PVD	30 (3.3)	60 (5.3)	0.025
Prior MI	166 (18)	215 (19)	0.595
Prior PCI	192 (20.9)	220 (19.4)	0.408
Prior CABG	105 (11.4)	146 (12.9)	0.315
Prior CVA	33 (3.6)	47 (4.1)	0.516
Prior TIA	19 (2.1)	33 (2.9)	0.226
STEMI	449 (48.8)	533 (47)	0.416
NSTEMI	458 (49.8)	598 (52.7)	0.183
GRACE Risk Score	98.02 (29.1)	101.23 (29.8)	0.014
In-hospital revascularization			
CABG	85 (9.2)	116 (10.2)	0.453
PCI	661 (71.8)	791 (69.8)	0.300
Discharge Medications			
Aspirin	793 (95.1)	1170 (95.9)	0.376
Clopidogrel	672 (80.6)	952 (78.0)	0.164
Aspirin + Clopidogrel	713 (77.5)	862 (76)	0.428
Beta Blocker	842 (91.5)	1039 (91.6)	0.935
ACE/ARB	689 (74.9)	829 (73.1)	0.359
Lipid Lowering	836 (90.9)	1029 (90.7)	0.920

Values are mean $\pm$ SD or N (%).

Table 3.

Unadjusted and adjusted HRs for (Caucasian) HAP3 carriers versus non-carriers in TRIUMPH

Model	Hazard Ratio (95% CI)	p-value
Unadjusted	0.46 (0.30, 0.72)	5.70E-04
Adjusted for GRACE Risk Score, HxPVD and Sex	0.52 (0.34, 0.81)	3.91E-03
Adjusted for GRACE Risk Score, HxPVD, Sex, and Smoking Status	0.51 (0.33, 0.79)	2.93E-03

Table 4.

Unadjusted and adjusted HRs for (Caucasian) HAP3 carriers versus non-carriers in INFORM/PREMIER validation cohort

Model	Hazard Ratio (95% CI)	p-value
Unadjusted	0.19 (0.06, 0.62)	6.04E-03
Adjusted for GRACE Risk Score, and Sex	0.20 (0.06, 0.67)	9.13E-03
Adjusted for GRACE Risk Score, Sex, and Smoking Status	0.20 (0.06, 0.67)	9.30E-03