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MELANOCORTIN-1 RECEPTOR POLYMORPHISMS AND THE RISK OF COMPLICATED SEPSIS AFTER TRAUMA: A CANDIDATE GENE ASSOCIATION STUDY

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Abstract

Objective—To determine if melanocortin-1 receptor (*MC1R*) single nucleotide polymorphisms (SNPs) are associated with complicated sepsis after trauma.

Background—Nosocomial infections are an important cause of morbidity and mortality after trauma. Several SNPs in inflammation-related genes have been associated with sepsis. *MC1R* is an anti-inflammatory mediator that may be involved in the immune response after trauma.

Methods—We genotyped 8 common *MC1R* SNPs in genomic DNA from subjects enrolled in a previously reported prospective cohort study. Subjects were adult trauma patients admitted to the ICU at a Level I trauma center (2003–2005).

Results—A total of 1,246 subjects were included in the analysis. The majority were male (70%), severely injured (81%), and injured by a blunt mechanism (89%). Forty percent developed sepsis, and 23% developed complicated sepsis, which was defined as sepsis with organ dysfunction. In logistic regression analysis, with adjustments for age, sex, body mass index, injury severity score, red blood cell transfusion requirement, and mechanism of injury, the *MC1R*_{R163Q} variant (rs885479) was associated with a lower risk of developing complicated sepsis (OR_{adj}=0.48, 95% CI: 0.28–0.81, p=0.006). In a subgroup of 511 subjects with genome-wide SNP data, the association between the *MC1R*_{R163Q} variant and complicated sepsis remained significant after adjusting for genetic substructure (by principal components) and the above clinical factors (OR_{adj}=0.30, 95% CI: 0.13–0.70, p=0.005).

Conclusions—*MC1R*_{R163Q} is associated with a lower risk of complicated sepsis after trauma. Therapeutic targeting of *MC1R* may be beneficial for trauma patients at risk for complicated sepsis.

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Keywords

MC1R; SNP; Arg163Gln; rs885479; melanocyte stimulating hormone

Introduction

Sepsis after trauma has a complex pathophysiology that involves both the pathogenic microbe and the patient's individualized immune response. Several single nucleotide polymorphisms (SNPs) are known to influence a patient's susceptibility to and recovery from sepsis. Many of these SNPs are in inflammation-related genes(1), such as lipopolysaccharide binding protein(2), interleukin-1(3), CD14(4), toll-like receptors(5, 6), and TNF- β (7). By understanding genetic variation that influences response to injury, we will move closer to personalized, or "precision medicine" - improving our ability to deliver the right treatment to the right patient at the right time.

The melanocortin-1 receptor (MC1R) is an anti-inflammatory mediator(8). It is a G protein-coupled receptor that binds with equal affinity to α -melanocyte stimulating hormone (α -MSH) and adrenocorticotrophic hormone (ACTH)(8). The receptor is expressed on numerous cells involved in the inflammatory response, including neutrophils, leukocytes, macrophages, endothelial cells, and melanocytes. Activation of the receptor results in a wide range of anti-inflammatory effects, including attenuation of macrophage cytokine production(9), reduction of leukocyte adhesion and migration(10), and inhibition of neutrophil chemotaxis(11).

The *MC1R* gene is highly polymorphic, with many functional variants(12). Several *MC1R* SNPs are associated with diseases such as melanoma(13), non-melanoma skin cancers(14), and multiple sclerosis(15). We previously reported that the *MC1R*_{R163Q} variant was associated with a higher risk of hypertrophic scarring after burn injury(16). Given the immunologic role of MC1R, we hypothesized that its common functional SNPs (Figure 1) would be associated with the risk of developing complicated sepsis after trauma.

Methods

Study design, population, and setting

We performed a retrospective cohort study using samples and data from a prior Institutional Review Board (IRB)-approved prospective study that enrolled subjects admitted to the trauma intensive care unit (ICU) at a regional Level 1 trauma center, between 2003 and 2005, as previously described(5, 17). Patients were excluded if they were in the ICU < 48 hours, had an isolated traumatic brain injury, or were expected to die from their injuries. Injury severity score (ISS) was obtained from a prospectively acquired trauma registry. Admission and clinical data were acquired from electronic medical records with IRB approval.

Exposures and outcomes

The exposures of interest were one of eight different *MC1R* SNP genotypes. Assessed outcomes included a) lower respiratory tract infection, defined as a quantitative protected bronchial culture demonstrating 10^5 colony forming units/ml; b) bacteremia, defined as bacterial growth in a blood culture; c) urinary tract infection, defined as a urine culture with greater than 10^5 organisms per high power field; d) wound infections, defined by clinical diagnosis with culture confirmation when available; and e) complicated sepsis, defined as severe sepsis or septic shock according to criteria established by The American College of Chest Physicians / Society of Critical Care Medicine Consensus Committee(18).

MC1R Genotyping

DNA was isolated from discarded venous blood with QIAamp® DNA Blood Midi Kits (Qiagen, Valencia, CA). Genotyping was performed on 20 ng DNA samples using pre-designed TaqMan® SNP Genotyping Assays (Qiagen) in 384-well plates with a Viia™7 Real-Time PCR System (Applied Biosystems, Foster City, CA) per manufacturer guidelines. The samples were genotyped for the following *MC1R* SNPs: *MC1R*_{V60L} (rs1805005), *MC1R*_{D84E} (rs1805006), *MC1R*_{V92M} (rs2228479), *MC1R*_{R142H} (rs11547464), *MC1R*_{R151C} (rs1805007), *MC1R*_{R163Q} (rs885479), *MC1R*_{D294H} (rs1805009), and *MC1R*_{T314T} (rs2228478) (Fig 1). SNPs with a minor allele frequency (MAF) $\geq 1\%$ were excluded from the analysis.

Genome-wide genotyping and principal components analysis

A subgroup of 511 Caucasian subjects had genome-wide genotyping data available from a previous study that investigated the genetic risks for ventilator-associated pneumonia (Wurfel and O'Keefe, manuscript in preparation). These subjects had required mechanical ventilation for ≥ 2 days. We did not test the SNPs from this genome-wide data for associations with complicated sepsis; instead, we used these SNPs as “null markers” of population substructure(19). The “null markers” were transformed into principal components, and the principal components were included as covariates in the regression analyses.

We determined the genotypes for approximately 620,901 polymorphisms. Each chip was stained and imaged on an Illumina Bead Array Reader. Normalized intensity data for each sample were loaded into Illumina Beadstudio 2.0 and genotypes called using the manufacturer's clustering algorithm. Dataset quality control was performed using SNP and Variation Suite 7.2 (Golden Helix, Bozeman, MT) and PLINK(20). We followed a published protocol for quality control in genetic case-control association studies(21). Samples with gender discordance as determined by X heterozygosity, excess relatedness, and those with an autosomal heterozygosity rate ± 3 standard deviations from the mean were excluded. Genetic markers were then filtered for call rate < 0.95 , minor allele frequency < 0.05 , and for deviations from Hardy-Weinberg equilibrium in the control population (HWE, $p < 0.005$). A total of 480,811 SNPs passed quality control filtering. We then performed principal components (PC) analysis to determine eigenvalues.

Statistical analysis

Associations between the *MC1R* SNP genotypes and outcomes were evaluated with univariate and multivariate logistic regression. We assumed any contribution to the sepsis phenotype from individual *MC1R* SNPs would be additive. Therefore, genotypes were coded as 0 (common allele homozygous), 1 (heterozygous), or 2 (variant allele homozygous). A univariate and multivariate model was fit for each SNP individually. Multivariate models included the following covariates: age and body mass index (BMI), modeled as continuous variables; sex, injury severity (ISS < 15 or ISS ≥ 15), and red blood cell (PRBC) transfusion requirement (<10 or ≥ 10 units PRBC transfusion during hospitalization) modeled as binary variables; and mechanism of injury (blunt, penetrating, or burn) modeled as a categorical variable. Hardy-Weinberg Equilibrium (HWE) was determined by χ^2 test. In the subgroup of subjects with genome-wide data, eigenvalues for the first 10 principal components (see above) were included in the model to adjust for population substructure.

In order to account for multiple testing, all p-value thresholds for significance were adjusted by the Bonferroni correction to control the familywise error rate at 0.05. Logistic regression analysis was performed using Stata® 11.2 statistical software (StataCorp LP, College Station, TX). Haplotype estimation and linkage disequilibrium analyses were performed using the Haplo Stats and LDheatmap packages, respectively, in R version 3.0.2.

Results

Demographics and Clinical Outcomes

Figure 2 diagrams the cohorts we analyzed and that are described below. In total, 1,961 subjects were enrolled (Supplemental Table 1). To minimize confounding by population substructure, only Caucasian subjects with complete clinical and *MC1R* genotyping data were included in the analysis (n=1,246). The demographics and clinical outcomes of this cohort are detailed in Table 1. The majority were male (70%), severely injured (81%), and injured by a blunt mechanism (89%). Four-hundred and ninety-seven (40%) developed sepsis and 290 (23%) developed complicated sepsis. Overall mortality was 9%, and this was higher among subjects with sepsis (11%) and complicated sepsis (14%).

Characterization of variation and haplotype structure of the *MCR1* gene

The technical genotyping success rate was 99% for all 8 *MC1R* SNP genotyping assays. Genotype frequencies and minor allele frequencies (MAF) among Caucasians with complete clinical data (N=1,246) are described in Table 2. *MC1R*_{V60L} was the most common SNP with a minor allele frequency (MAF) of 12%. *MC1R*_{R163Q} had a minor allele frequency of 6%. *MC1R*_{D84E} and *MC1R*_{R142H} had MAFs < 1%, and were therefore excluded from further analysis, leaving 6 SNPs (*MC1R*_{V60L}, *MC1R*_{V92M}, *MC1R*_{R151C}, *MC1R*_{R163Q}, *MC1R*_{D294H}, and *MC1R*_{T314T}) for association testing. In haplotype analysis only *MC1R*_{V92M} and *MC1R*_{T314T} had a relevant degree of linkage disequilibrium ($R^2=0.74$) (Fig 3), with a *MC1R*_{V92M}/*MC1R*_{T314T} haplotype frequency of 8.4%.

The *MC1R*_{R163Q} variant was associated with a lower risk of complicated sepsis

The incidence of complicated sepsis varied by genotype, ranging from 9% among *MC1R*_{163QQ} homozygotes to 50% among *MC1R*_{151CC} homozygotes. Unadjusted and adjusted analyses of associations between the *MC1R* SNPs and complicated sepsis are shown in Table 3. Six SNPs were analyzed, so the Bonferroni-adjusted p-value threshold for significance was 0.05/6=0.008. In univariate analysis, the *MC1R*_{R163Q} variant was associated with a significantly lower risk of complicated sepsis (OR=0.42, 95% CI: 0.26–0.69, p=0.001). This association persisted after adjusting for age, sex, ISS, PRBC transfusion requirement, BMI, and mechanism of injury (OR_{adj}=0.48, 95% CI: 0.28–0.81, p=0.006) (Table 3). There were no associations between the other *MC1R* SNPs and complicated sepsis in univariate or multivariate analyses. In addition, there were no associations between complicated sepsis and *MC1R* haplotypes that did not include the *MC1R*_{R163Q} variant. Notably, the *MC1R*_{R163Q} variant was not associated with the risk of developing an infection (OR_{adj}=0.78, 95% CI: 0.56–1.09, p=0.142) or sepsis without organ dysfunction (OR_{adj}=0.77, 95% CI: 0.54–1.11, p=0.158).

The *MC1R*_{R163Q} variant was associated with a lower risk of complicated sepsis after adjusting for population substructure

All SNPs were in Hardy-Weinberg Equilibrium (HWE) (p>0.05), with the exceptions of *MC1R*_{R163Q} (p=0.005) and *MC1R*_{D294H} (p<0.001). The departure from HWE equilibrium raised that possibility that the association between *MC1R*_{R163Q} and complicated sepsis was an artifact of population substructure. We used principal components to adjust for this potential substructure in a subset of subjects (n=511) who had genome-wide data from a previous study (Wurfel and O’Keefe, manuscript in preparation). The characteristics of this subgroup are detailed in Supplemental Table 1. In regression analysis, principal components did not attenuate the association between the *MC1R*_{R163Q} variant and lower risk of complicated sepsis (OR_{adj}=0.30, 95% CI: 0.13–0.70, p=0.005).

Discussion

Our results suggest that variation in the *MC1R* gene is associated with the severity of sepsis after traumatic injury. Subjects with the *MC1R*_{R163Q} variant had a lower risk of developing complicated sepsis. The variant did not appear to influence the risk of developing an infection, but rather appeared to influence the host response to a nosocomial infection. This is consistent with other studies of the relationship between genetic variation and infection severity(22).

The mechanism for the inverse association between the *MC1R*_{R163Q} variant and complicated sepsis is unclear, but this SNP may be a causal factor behind the improved outcomes. Several previous studies showed that the melanocortin system mediates inflammation and plays a role in sepsis. In an *in vivo* study, mice with defective MC1R signaling had more severe vascular dysfunction in response to lipopolysaccharide (LPS) than did wild-type mice(23). In another study, mice treated with α -MSH had less liver damage in response to LPS compared to untreated mice, an effect that was mediated by decreased leukocyte infiltration and cytokine accumulation(24). In mice with sepsis induced by cecal ligation and

perforation, an α -MSH analogue decreased hemodynamic failure, acute kidney injury, and mortality(25). Moreover, α -MSH has been associated with the inflammatory response and critical illness in humans(26). In healthy subjects, endotoxin administration resulted in increased serum α -MSH concentrations(27). In critically-ill subjects with sepsis, higher serum α -MSH concentrations were associated with lower serum TNF- α concentrations, and septic patients with persistently low α -MSH concentrations had a higher risk of death(26). Taken together, these studies indicated that the melanocortin system participates in the innate immune response and that experimental genetic variation alters responses. Therefore, it is possible that the association we observed between an MC1R variant and complicated sepsis is due to a functional change in the receptor.

MC1R signaling involves two pathways: a cyclic AMP (cAMP) pathway that is activated by adenylate cyclases(28), and a cAMP-independent pathway involving mitogen-activated protein kinases (MAPK)(29). In general, these two pathways elicit different responses by the immune system. The MAPK pathway, which is also activated by TNF- α receptors and toll-like receptors(30), has pro-inflammatory effects (30, 31), in part by inducing expression of TNF- α (32). The cAMP pathway, on the other hand, elicits an anti-inflammatory response(33), by decreasing expression of pro-inflammatory cytokines, such as TNF- α (34) and macrophage inflammatory protein-1 α (35), and by increasing expression of anti-inflammatory cytokines, such as IL-10(36).

The *MC1R*_{R163Q} variant, which substitutes a charged arginine residue with a polar glutamine residue in the receptor's GTPase domain(37), is not a simple loss- or gain-of-function mutation. In an *in vitro* study using human embryonic kidney cells, Doyle et al.(38) found that the *MC1R*_{R163Q} variant resulted in a loss of MAPK signaling but preserved cAMP signaling. This is distinct from several other *MC1R* variants (*MC1R*_{R151C}, *MC1R*_{R160W}, *MC1R*_{D294H}) that had normal MAPK activation but attenuated cAMP signaling(29, 39). Therefore, the *MC1R*_{R163Q} variant may lower the risk of a severe systemic response to infection by changing the balance between pro- and anti-inflammatory signals from the MC1R receptor.

It is possible that the *MC1R*_{R163Q} variant is not causally linked to a lower risk of complicated sepsis; rather the observed association may be due to one of three genetic mechanisms. First, the minor allele frequencies of *MC1R* SNPs differ greatly between populations(12), so any association between them and a phenotype may be an artifact of population substructure; individuals with the *MC1R*_{R163Q} variant may have a high degree of common ancestry with many shared variants, any of which may be the true causal variant. We adjusted for population substructure among subjects with available genome-wide data and found that the association was not attenuated, which suggested that the association was not due to population substructure.

Second, other haplotypes may have increased the risk of complicated sepsis. Currently there is debate about how genetic variants contribute to common complex diseases, with two main hypotheses. The common disease - common variant (CD-CV) hypothesis proposes that many common variants with low penetrance determine genetic susceptibility to common diseases. Alternatively, the common disease - rare variant (CD-RV) hypothesis proposes that

multiple rare variants with relatively higher penetrance determine genetic susceptibility to these diseases. Based on the CD-RV hypothesis, it is possible that rare high-risk variants, which we did not test and may have been multiple, were more common in haplotypes that did not contain the *MC1R*_{R163Q} variant. In this scenario, the *MC1R*_{R163Q} variant was not responsible for the lower incidence of complicated sepsis, but served as a marker for individuals who did not have a high risk haplotype. Finally, the *MC1R*_{R163Q} variant may have tagged a haplotype that included the true causal variant.

The findings from this study have several potential implications. They further elucidate the role of genetic risk in the pathobiology of sepsis. Genotyping has yet to become a component of clinical practice in trauma care, but increasing our understanding of genetic variants has potential to improve risk stratification and influence clinical decisions(40), such as antibiotic use and resuscitation. In addition, if the functional effects of the *MC1R*_{R163Q} variant are confirmed, it may help direct future drug development. Currently, customized immunomodulatory therapy for sepsis is lacking: recombinant activated protein C, chimeric antibodies against TNF- α , and glucocorticoids have all failed to show a clear survival benefit. Biased agonists that selectively change the signaling pattern of MC1R(41) may be worthwhile alternatives to investigate. An agonist that manipulates MC1R signaling in a manner similar to the *MC1R*_{R163Q} variant could reduce the risk of a severe inflammatory response after trauma or infection.

This study had several limitations. Like all candidate gene association studies, it may be confounded by population substructure, which was not fully adjusted for in the main analysis. In keeping with the accepted approach to genetic association studies, we restricted our analyses to one race/ethnicity (in this case Caucasians, our largest cohort) so our findings cannot be generalized to other races/ethnicities. Another potential concern is related to the recently updated definitions of sepsis severity by the Sepsis Definitions Task Force, convened by the Society of Critical Care Medicine and the European Society of Intensive Care Medicine(42). According to the new definition, sepsis is an infection with associated organ dysfunction, defined as an acute change in Sequential Organ Failure Assessment (SOFA) score ≥ 2 points. SIRS criteria were excluded from the definition because they were neither sensitive nor specific to sepsis. Our current study measured organ dysfunction using the Multiple Organ Dysfunction Score rather than the SOFA score. However, our definition of “complicated sepsis” incorporates both sepsis and septic shock as defined in the Sepsis-3 definitions. Finally, our new finding of an association between *MC1R* and complicated sepsis requires confirmation in an independent sample of trauma patients before using the gene for screening, risk stratification, or therapeutic targeting.

In conclusion, our study suggested that MC1R has a role in the pathophysiology of sepsis after trauma. The *MC1R*_{R163Q} variant was associated with a lower risk of complicated sepsis in severely injured trauma patients. This SNP has the potential to be used in “precision medicine” for risk stratification and clinical decision making. Finally, therapeutic targeting of the MC1R pathway may be beneficial for trauma patients who are at high risk for developing complicated sepsis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MC1R protein structure

The receptor has 7 transmembrane domains. Eight of its common functional SNPs are indicated by their substituted amino acids and location in the peptide chain.

Figure 2. Subject enrollment for MC1R genotyping study

Caucasian subjects with complete clinical and *MC1R* genotyping data (N=1,246) were included in the main analysis. A subgroup analysis (N=511) was performed on Caucasian subjects who had data available from a previous genome-wide association study (GWAS; Wurfel and O’Keefe, manuscript in preparation).

Figure 3. Pair-wise linkage disequilibrium heat map for the six MC1R SNPs included in the haplotype analysis
 R^2 is the square of the correlation coefficient between the two SNPs.

Table 1

Subject demographics, characteristics, and outcomes (N = 1,246).

Characteristics	N (%) or median (IQR)
Age, years	39 (22–53)
Male	869 (70%)
Injury severity score 15	1,013 (81%)
10 units PRBC transfusion	176 (14%)
BMI, kg/m ²	26 (22–29)
Mechanism of injury	
Blunt	1,107 (89%)
Penetrating	76 (6%)
Burn	63 (5%)
Outcomes	N (%)
Infections	
Any infection	600 (48%)
Urinary tract infection	237 (19%)
Lower respiratory tract infection	155 (12%)
Wound infection	150 (12%)
Bacteremia	167 (13%)
Sepsis	497 (40%)
Complicated sepsis	290 (23%)
Mortality	
Overall	113 (9%)
Among subjects with sepsis	54 (11%)
Among subjects with complicated sepsis	42 (14%)

Continuous variables are reported as median (IQR) and binary variables are reported as number (%).

Table 2*MC1R* SNP genotypes and minor allele frequencies.

<i>MC1R</i> SNP	Amino acid substitution	rs identifier	Successfully genotyped (N)	Common allele homozygous (N) ^a	Heterozygous (N) ^a	Variant allele homozygous (N) ^a	Minor Allele Frequency (%) ^b
<i>MC1R</i> _{V60L}	Val60Leu	rs1805005	1,246	962	265	19	12%
<i>MC1R</i> _{D84E}	Asp84Glu	rs1805006	1,244	1,216	28	0	1%
<i>MC1R</i> _{V92M}	Val92Met	rs2228479	1,246	1,042	198	6	8%
<i>MC1R</i> _{R142H}	Arg142His	rs11547464	1,246	1,231	15	0	1%
<i>MC1R</i> _{R151C}	Arg151Cys	rs1805007	1,244	1,057	179	8	8%
<i>MC1R</i> _{R163Q}	Arg163Gln	rs885479	1,246	1,098	137	11	6%
<i>MC1R</i> _{D294H}	Asp294His	rs1805009	1,245	1,204	34	7	2%
<i>MC1R</i> _{T314T}	Thr314Thr	rs2228478	1,246	979	258	9	11%

^aNumber of subjects with each corresponding genotype.^bMinor allele frequency (MAF) was calculated by the equation $MAF = ((2 \times \text{SNP homozygotes}) + (\text{Heterozygotes})) / (2N)$.

Table 3

The *MC1R*_{R163Q} variant is inversely associated with complicated sepsis.

<i>MC1R</i> SNP	Cases of complicated sepsis, N (%) ^a			Univariate Analysis ^b		Multivariate Analysis ^b	
	Common allele homozygous	Heterozygous	Minor allele homozygous	OR for complicated sepsis (95% CI)	p-value	OR _{adj} ^c for complicated sepsis (95% CI)	p-value
<i>MC1R</i> _{V60L}	216 (22%)	66 (25%)	8 (42%)	1.25 (0.95–1.65)	0.097	1.27 (0.94–1.72)	0.122
<i>MC1R</i> _{V92M}	247 (24%)	41 (21%)	2 (33%)	0.88 (0.63–1.26)	0.502	0.85 (0.58–1.25)	0.404
<i>MC1R</i> _{R151C}	246 (23%)	39 (22%)	4 (50%)	1.05 (0.75–1.48)	0.765	0.99 (0.68–1.44)	0.947
<i>MC1R</i> _{R163Q}	273 (25%)	16 (12%)	1 (9%)	0.42 (0.26–0.69)	0.001 ^d	0.48 (0.28–0.81)	0.006 ^d
<i>MC1R</i> _{D294H}	280 (23%)	9 (27%)	1 (14%)	0.98 (0.54–1.79)	0.956	1.01 (0.53–1.96)	0.968
<i>MC1R</i> _{T314T}	225 (23%)	63 (24%)	2 (22%)	1.07 (0.79–1.44)	0.668	1.07 (0.77–1.49)	0.696

^aNumber of subjects with complicated sepsis, by genotype (incidence of complicated sepsis among subjects with the corresponding genotype (%)).

^bUnivariate and multivariate logistic regression with *MC1R* SNP as the independent variable and complicated sepsis as the outcome variable. Each SNP was analyzed individually in the regression model.

^cAdjusted for age, sex, injury severity score (<15 or ≥15), PRBC transfusion requirement (<10 or ≥10 units), BMI, and mechanism of injury (blunt, penetrating, or burn).

^dSignificant association below the Bonferroni-adjusted p-value threshold (p = 0.008).