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Heme Oxygenase-1 microsatellite polymorphism and haplotypes are associated with the development of acute respiratory distress syndrome

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Abstract *Objective:* Heme oxygenase-1 (HO-1) acts in cytoprotection against acute lung injury. The polymorphic $(GT)_n$ repeat in the HO-1 gene (*HMOX1*) promoter regulates *HMOX1* expression. We investigated the associations of *HMOX1* polymorphisms with acute respiratory distress syndrome (ARDS) risk and plasma HO-1 levels. *Design:* Unmatched, nested case–control study. *Setting:* Academic medical center. *Patients:* Consecutive patients with ARDS risk factors upon ICU admission were prospectively enrolled. Cases were 437 Caucasians who developed ARDS and controls were 1,014 Caucasians who did not. *Measurements and results:* We genotyped the $(GT)_n$ polymorphism and three tagging single nucleotide polymorphisms (tSNPs) in 1,451 patients, and measured the plasma HO-1 levels in 106 ARDS patients. We clustered the $(GT)_n$ repeats into: S-allele (<24 repeats), M-allele (24–30 repeats) and L-allele (≥31 repeats). We found that longer $(GT)_n$ repeats were associated with reduced ARDS risk ($P_{trend} = 0.004$ for both alleles and genotypes), but no individual tSNP was associated with ARDS risk. *HMOX1* haplotypes were significantly associated with ARDS risk (global test, $P = 0.016$), and the haplotype S-TAG was associated with increased ARDS risk (OR, 1.75; 95% CI, 1.15–2.68; $P = 0.010$). Intermediate-phenotype analysis showed longer $(GT)_n$ repeats were associated with higher plasma HO-1 levels ($P_{trend} = 0.019$ for alleles and 0.027 for genotypes). *Conclusions:* Longer $(GT)_n$ repeats in the *HMOX1* promoter are associated with higher plasma HO-1 levels and reduced ARDS risk. The common haplotype S-TAG is associated with increased ARDS risk. Our results suggest that *HMOX1* variation may modulate ARDS risk through the promoter microsatellite polymorphism.

Keywords Acute respiratory distress syndrome · Genetic susceptibility · Haplotypes · Heme oxygenase-1 · Microsatellite polymorphism · Molecular epidemiology

Introduction

Acute respiratory distress syndrome (ARDS) is a common and devastating disease in intensive care units (ICUs) worldwide, with a high mortality rate of 35–40% [1]. Recently, there is increasing evidence that heritable susceptibility may explain at least part of the risk of developing ARDS after pulmonary or extrapulmonary insults.

Heme oxygenase-1 (HO-1) is an inducible isoform of HO, which catalyzes the degradation of heme, a pro-oxidant, into carbon monoxide, biliverdin, and iron [2]. Biliverdin is subsequently converted to bilirubin, and iron is sequestered by ferritin. These metabolites of heme catabolism have antioxidant, anti-apoptotic, anti-proliferative and anti-inflammatory effects; therefore, HO-1 has emerged as an important cytoprotective enzyme in the human body [3, 4].

Oxidant–antioxidant imbalance is important in the complex pathogenesis of ARDS [5, 6]. Expression of the antioxidant enzyme HO-1 is upregulated in animal models and human lungs of ARDS [7, 8]. Animal studies showed that HO-1 plays a protective role against acute lung injury induced by hyperoxia, ischemia–reperfusion, and endotoxin [3, 9]. Overexpression of HO-1 by gene transfer protects rat lungs against hyperoxia-induced lung injury [10]. Induction of HO-1 by hemoglobin protects rat lungs against hyperoxia- [11] and endotoxin-induced lung injury [12]. In a nebulized endotoxin-induced lung injury model, HO-1-deficient mice develop severe lung dysfunction with marked reduction in surfactant protein B levels [13]. In addition, the HO-1-deficient mice exhibit lethal ischemia-reperfusion lung injury but could be rescued from death by inhaled carbon monoxide [14]. To date, evidence has accumulated for the protective effects and therapeutic potential of HO-1, as well as the downstream metabolites, in acute lung injury [15].

The HO-1 gene (*HMOX1*) is located on chromosome 22q13.1. A polymorphic $(GT)_n$ repeat in the *HMOX1* promoter regulates the promoter activity and gene expression [16–19]. Studies showed that the longer $(GT)_n$ repeats are associated with increased susceptibility to pulmonary diseases such as smoking-induced emphysema [19], pneumonia [20], and asthma [21]. This microsatellite polymorphism has also been associated with a variety of diseases, including coronary artery disease, renal transplantation [3], idiopathic recurrent miscarriage [22], cerebral malaria [23], and rheumatoid arthritis [18].

In this study, we hypothesized that *HMOX1* variation may influence susceptibility to ARDS. We genotyped the $(GT)_n$ polymorphism and the tagging single nucleotide polymorphisms (tSNPs) of *HMOX1*, and used an unmatched, case–control design to analyze the association of *HMOX1* polymorphisms with ARDS development. We also measured the plasma HO-1 levels in a subset of ARDS patients to assess the functional significance of *HMOX1* polymorphisms.

Patients and methods

Study population

Study patients were recruited in the ICUs at Massachusetts General Hospital, Boston, from September 1999 to November 2006. Definitions and details of the study design have been described previously [24]. Briefly, all consecutive admissions to the ICUs were screened. Patients with clinical risk factors for ARDS were eligible for inclusion (see electronic supplementary material, ESM1, for details of the inclusion and exclusion criteria). Baseline characteristics and the acute physiology and chronic health evaluation (APACHE) III scores were recorded on ICU admission. The enrolled patients were screened daily for ARDS development and those who fulfilled the American-European Consensus Committee (AECC) criteria for ARDS [25] were considered as ARDS cases, whereas at-risk patients who did not develop ARDS during ICU stay were considered as controls. We restricted our analysis to non-Hispanic Caucasians (91.8% in our study population). The study was approved by the Human Subjects Committees of the Massachusetts General Hospital and the Harvard School of Public Health. Written informed consent was obtained from all subjects or surrogates.

Tagging SNPs selection

We selected the tSNPs across the entire *HMOX1* gene, including 2 kbp on each side. We used the multimarker tagging algorithm with criteria of minor allele frequency ≥ 0.1 and $r^2 > 0.8$, based on the HapMap data (release 23a) of the CEU population (Utah residents with ancestry from northern and western Europe). Because rs2071746 (−413A>T) has been related to the promoter function, this SNP was forced in as a tSNP during the tagging process. As a result, we identified a set of three tSNPs that efficiently tagged all known common *HMOX1* variants.

Genotyping

Genomic DNA was extracted from whole blood using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) or by the AutoPure LS robotic workstation using the Autopure reagents (Qiagen, Valencia, California). The $(GT)_n$ polymorphism was genotyped by a PCR method in combination with fluorescence technology as previously described [19]. Briefly, the gene promoter

containing the $(GT)_n$ repeats was amplified with the labeled forward primer 5′-AGAGCCTGCAGCTTCTC AGA-3′ and the reverse primer 5′-ACAAAGTCTG GCCATAGGAC-3′. The sizes of the PCR products were then determined by Applied Biosystems 3730xl DNA analyzer and the GeneMapper software (Applied Biosystems, Foster City, CA). The tSNPs were genotyped by the TaqMan® SNP Genotyping Assay (Applied Biosystems). The primers and probes were ordered from Applied Biosystems. The fluorescence of PCR products was detected by the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). Genotyping was performed by laboratory personnel blinded to case–control status. A random 10% of samples were included as duplicates for quality-control. The overall genotyping success rate was >98% and the concordance rate for duplicate samples was >99%. Samples not yielding the genotypes of all polymorphisms were excluded from analysis.

Classification of $(GT)_n$ repeats

The standard classification of $(GT)_n$ repeats remains inconclusive. We performed a sensitivity analysis to determine the best cutoffs. The results showed that grouping the alleles into short (S)-allele (<24 repeats), middle (M)-allele (24–30 repeats), and long (L)-allele (≥31 repeats) was the best-fit model (ESM2). We also did a thorough literature review to find the most commonly used grouping criteria. These criteria were S-allele (<25 repeats) and L-allele (≥25 repeats) for two allele groups; and S-allele (<27 repeats), M-allele (27–32 repeats), and L-allele (≥33 repeats) for three allele groups (ESM3).

Plasma HO-1 measurement

Plasma HO-1 levels were determined in 106 ARDS patients with available plasma samples collected during the first 48 h after ARDS diagnosis. We used the Human HO-1 ELISA Kit (Assay Designs Inc., Ann Arbor, MI) and followed the manufacturer's assay procedure. This ELISA kit has been quantified for measurement of human blood HO-1 in previous studies [26–28]. All the samples were tested in duplicate.

Statistical analyses

Baseline characteristics between cases and controls were evaluated by χ^2 test for categorical variables and by unpaired t test for continuous variables. We used SAS/Genetics to calculate the allele frequencies, to test the deviation from Hardy–Weinberg equilibrium, and to analyze the pairwise D′ and r^2 values for linkage disequilibrium (LD). We also used SAS/Genetics to generate the maximum likelihood estimates of haplotype frequencies, using the expectation-maximization algorithm. These estimates were then used to assign the probability that each individual possessed a particular haplotype pair.

The differences of allele, genotype and haplotype frequencies between cases and controls were compared by χ^2 test. The associations between *HMOX1* polymorphisms and ARDS risk were analyzed in logistic regression models, adjusting for age, gender, severity score (modified APACHE III scores, which removed age and PaO_2/FiO_2 components to avoid colinearity), risk factors for ARDS (as inclusion criteria in this study), and other potential risks for ARDS based on univariate analysis. The genotype associations were analyzed in both codominant and additive models. Haplotypes with frequency >5% were considered as common haplotypes and rare haplotypes were pooled as a group. We used all other haplotypes as the reference to estimate the odds ratio (OR) and 95% confidence interval (CI) associated with a particular haplotype pair.

We used linear regression models to analyze the associations of clinical variables, genotypes and haplotypes with plasma HO-1, and to calculate the P values or P values for trend (P_{trend}). Prior to statistical analysis, plasma HO-1 was Box-Cox transformed to comply with normality. All data were analyzed by SAS, version 9.1 (SAS Institute, Cary, NC). A P value < 0.05 was considered statistically significant. For significant associations, false discovery rate (FDR) adjusted P values were calculated to correct for multiple testing.

Results

Study population

During the study period, 2,626 patients were eligible for enrollment. Nine hundred patients without consents and 96 patients with previous history of ARDS or previous enrollment as controls were not included. As a result, a total of 1,630 patients were enrolled into the prospective cohort. Among these participants, 134 non-Caucasians, seven without complete clinical data and 38 without complete genotyping data were excluded sequentially, leaving 1,451 patients for analysis. Cases were 437 (30.1%) patients who developed ARDS, and controls were 1,014 (69.9%) patients who did not develop ARDS. Their baseline characteristics are shown in Table 1.

Association of *HMOX1* $(GT)_n$ polymorphism with ARDS

The numbers of $(GT)_n$ repeats showed a bimodal distribution of 13–40, with two peaks at 23 and 30 repeats

Table 1 Baseline characteristics of study population

	Cases, patients developed ARDS ($n = 437$)	Controls, patients did not develop ARDS ($n = 1{,}014$)	P value
Age (years), mean ± SD	60 ± 18	63 ± 17	0.002
Male, *n* (%)	264 (60.4%)	614 (60.6%)	0.960
APACHE III score, mean ± SD	77 ± 24	67 ± 23	<0.001
Risk factors, *n* (%)			
Sepsis (without shock)	113 (25.9%)	376 (37.1%)	<0.001
Septic shock	261 (59.7%)	442 (43.6%)	<0.001
Pneumonia	296 (67.7%)	435 (42.9%)	<0.001
Aspiration	43 (9.8%)	86 (8.5%)	0.404
Multiple transfusion	45 (10.3%)	116 (11.4%)	0.525
Trauma	32 (7.3%)	78 (7.7%)	0.807
Comorbidity, *n* (%)			
Post-operative	28 (6.4%)	70 (6.9%)	0.730
Diabetes	78 (17.9%)	274 (27.0%)	<0.001
End-stage renal disease	27 (6.2%)	50 (4.9%)	0.331
Liver cirrhosis/failure	30 (6.9%)	38 (3.8%)	0.010
Metastatic cancer	13 (3.0%)	49 (4.8%)	0.109
History of steroid use	46 (10.5%)	91 (9.0%)	0.354
History of alcohol abuse	62 (14.2%)	101 (10.0%)	0.019

ARDS acute respiratory distress syndrome, *APACHE* acute physiology and chronic health evaluation

(Fig. 1). There was a trend that longer $(GT)_n$ repeats were associated with reduced risk of ARDS ($P_{trend} = 0.026$). Based on the results of sensitivity analysis, we divided the $(GT)_n$ repeats into: S-allele (<24 repeats), M-allele (24–30 repeats) and L-allele (≥31 repeats), which resulted in six genotypes: SS, SM, MM, SL, ML, and LL.

The allele and genotype frequencies of the $(GT)_n$ polymorphism are shown in Table 2. The genotype distribution among controls was in Hardy–Weinberg equilibrium ($P = 0.732$). We found significant differences of allele and genotype frequencies between ARDS and controls ($P = 0.006$ and $P = 0.033$, respectively). In multivariate analysis, longer $(GT)_n$ repeats were associated with lower risk of developing ARDS in a gene-dose dependent relationship ($P_{trend} = 0.004$ for both allele and genotype analyses). Individual genotypes of SM, MM, SL, and ML were also significantly associated with reduced ARDS risk ($P = 0.009$, 0.004, 0.013, and 0.0004, respectively), compared with the genotype SS. Genotype LL, however, did not reach statistical significance due to relatively small sample size.

Then we reclassified the $(GT)_n$ repeats according to other common grouping criteria. In the two-allele-group model, the genotype LL remained significantly associated with reduced ARDS risk, compared with genotype SS (OR, 0.63; 95% CI, 0.40–0.98; $P = 0.043$). In the three-allele-group model, the L-allele was associated with reduced ARDS risk, compared with S-allele (OR, 0.63;

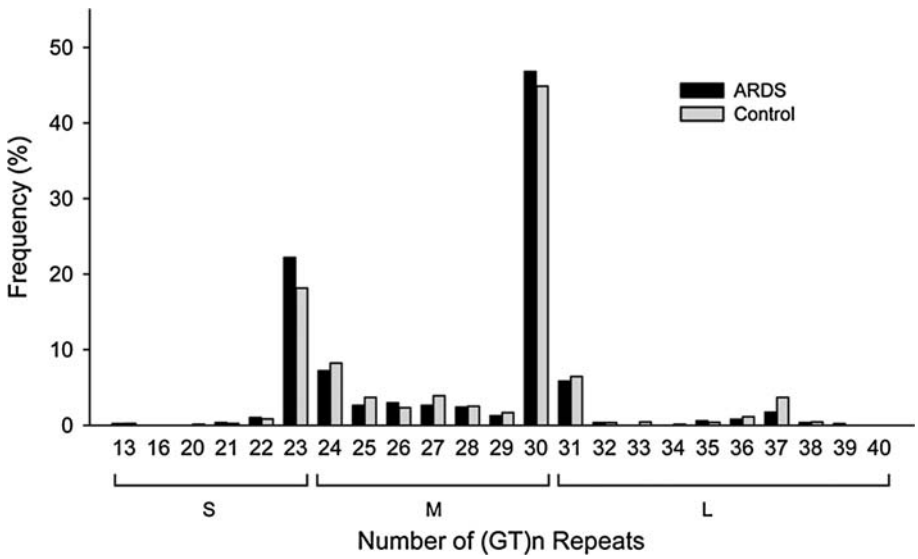

Fig. 1 Frequency distribution of $(GT)_n$ repeats in patients with ARDS (*black bar*, $n = 437$) and controls (*gray bar*, $n = 1{,}014$). The numbers of $(GT)_n$ repeats ranged from 13 to 40 and showed a bimodal distribution, with one peak located at 23 repeats and the other located at 30 repeats. The overall frequency distributions of $(GT)_n$ repeats were not significantly different between ARDS and controls (χ^2 test, $P = 0.085$). However, there was a trend that longer $(GT)_n$ repeats were associated with reduced risk of ARDS ($P_{trend} = 0.026$)

Table 2 Associations between *HMOX1* $(GT)_n$ polymorphism and acute respiratory distress syndrome risk

	Cases ($n = 437$)	Controls ($n = 1,014$)	Incidence of ARDS (%)	*P* value	Crude		Adjusted[b]		
					OR (95% CI)	*P* value	OR (95% CI)	*P* value	P_{trend}
Allele[a]				0.006*					0.004*
S-allele, <24 repeats	210	398	34.1		1.00		1.00		
M-allele, 24–30 repeats	576	1,363	29.4		0.80 (0.66–0.98)	0.027	0.81 (0.66–0.99)	0.045	
L-allele, ≥31 repeats	88	267	24.6		0.63 (0.47–0.85)	0.002	0.66 (0.47–0.88)	0.004	
Genotype				0.033					0.004*
SS	28	36	43.8		1.00		1.00		
SM	130	269	32.6		0.62 (0.36–1.06)	0.082	0.46 (0.26–0.83)	0.009	
MM	194	465	29.9		0.55 (0.33–0.92)	0.023	0.44 (0.25–0.78)	0.004	
SL	24	57	29.6		0.54 (0.27–1.08)	0.080	0.39 (0.19–0.82)	0.013	
ML	58	182	24.2		0.41 (0.23–0.73)	0.002	0.33 (0.18–0.61)	0.0004	
LL	3	14	17.7		0.28 (0.07–1.05)	0.060	0.34 (0.08–1.38)	0.131	

HMOX1 heme oxygenase-1 gene, *ARDS* acute respiratory distress syndrome, *CI* confidence interval, *OR* odds ratio, *S* short, *M* middle, *L* long

* FDR adjusted *P* value < 0.05

[a] In allele analysis, $n = 858$ for ARDS and 2,024 for controls

[b] Adjusted for age, gender, APACHE III score, all risk factors for ARDS (listed in Table 1), diabetes, liver cirrhosis/failure and alcohol abuse. APACHE III score was revised to remove age and PaO_2/FiO_2 to avoid colinearity

95% CI, 0.41–0.96; $P = 0.032$); and the genotypes with L-allele (SL + ML + LL) were also associated with a reduced ARDS risk, compared with the genotypes without L-allele (SS + SM + MM) (OR, 0.64; 95% CI, 0.42–0.98; $P = 0.040$) (ESM4).

Association of *HMOX1* tSNPs with ARDS

The alleles, locations, chromosome positions, minor allele frequencies, Hardy–Weinberg equilibrium, and pairwise LD of the three tSNPs (rs2071746, rs2071748 and rs5755720) are summarized in ESM5. All tSNPs conformed to Hardy–Weinberg equilibrium and were in high LD with each other ($D' \geq 0.982$). Only one LD block in *HMOX1* gene was found (ESM6). Further LD analysis showed that minor alleles of all three tSNPs were in LD with the S-allele of $(GT)_n$ polymorphism (ESM7).

The minor allele and genotype frequencies were not different between ARDS and controls for all tSNPs (Table 3). None of the individual tSNPs showed a significant association with ARDS risk.

Association of *HMOX1* haplotypes with ARDS

Based on the $(GT)_n$ polymorphism and tSNPs, 16 haplotypes were found. Five of them were common haplotypes with frequencies >5%. The haplotypes were expressed in the order of $(GT)_n$ polymorphism, rs2071746, rs2071748 and rs5755720, and their frequencies in ARDS patients and controls are shown in Table 4. The estimated frequency of haplotype S-TAG, which carried the short $(GT)_n$ repeats and minor alleles of all tSNPs, in ARDS patients was significantly higher than that in controls (22.5 vs. 18.0%, $P = 0.005$). The global test showed a significant association between *HMOX1* haplotypes and ARDS risk in both univariate and multivariate analyses (likelihood ratio test, $P = 0.005$ and 0.016, respectively). In haplotype-specific analysis, haplotype S-TAG was significantly associated with increased risk of developing ARDS (OR, 1.75; 95% CI, 1.15–2.86; $P = 0.010$).

Correlations of clinical variables and *HMOX1* polymorphisms with plasma HO-1 levels in ARDS patients

There were no significant differences of baseline characteristics, ARDS outcomes, and genotype distributions between ARDS patients with or without plasma HO-1 measured, except for APACHE III scores and history of diabetes (ESM8 and ESM9). None of the demographic or clinical factors was independently associated with plasma HO-1 levels during acute-phase of ARDS (ESM10).

The plasma HO-1 levels among different *HMOX1* genotypes in ARDS patients are shown in Fig. 2. We found that longer $(GT)_n$ repeats were associated with higher plasma HO-1 levels ($P_{trend} = 0.019$ for allele analysis and 0.027 for genotype analysis). Although patients with variant homozygotes of the three tSNPs had lower plasma HO-1 levels than those with wildtype alleles, the differences did not reach statistical significance. In haplotype analysis, only one common haplotype, L-AGA, was positively associated with plasma HO-1 levels ($P = 0.002$) (Table 5).

Table 3 Association between *HMOX1* tSNPs and acute respiratory distress syndrome risk

tSNPs	Cases ($n = 437$)	Controls ($n = 1{,}014$)	Incidence of ARDS (%)	*P* value	Crude		Adjusted[a]		
					OR (95% CI)	*P* value	OR (95% CI)	*P* value	P^{b}_{trend}
Alleles, minor allele (MAF)									
rs2071746, T (42.6%)	360	867	29.3	0.663	0.97 (0.82–1.13)	0.663	0.96 (0.81–1.14)	0.649	
rs2071748, A (38.0%)	333	759	30.5	0.507	1.06 (0.90–1.25)	0.506	1.03 (0.86–1.23)	0.745	
rs5755720, G (30.6%)	278	604	31.5	0.173	1.13 (0.95–1.34)	0.173	1.08 (0.90–1.29)	0.437	
Genotypes									
rs2071746				0.882					0.688
AA	148	330	31.0		1.00		1.00		
AT	210	499	29.6		0.94 (0.73–1.21)	0.621	0.90 (0.69–1.18)	0.433	
TT	79	185	29.9		0.95 (0.69–1.32)	0.769	0.96 (0.67–1.36)	0.807	
rs2071748				0.524					0.686
GG	164	388	29.7		1.00		1.00		
AG	205	491	29.5		0.99 (0.77–1.26)	0.922	0.90 (0.69–1.17)	0.420	
AA	68	135	33.5		1.19 (0.85–1.68)	0.318	1.18 (0.82–1.71)	0.382	
rs5755720				0.222					0.337
AA	200	494	28.8		1.00		1.00		
AG	189	436	30.2		1.07 (0.85–1.36)	0.572	0.99 (0.77–1.27)	0.911	
GG	48	84	36.4	0.101	1.41 (0.96–2.09)	0.084	1.36 (0.89–2.06)	0.153	

HMOX1 heme oxygenase-1 gene, *ARDS* acute respiratory distress syndrome, *tSNP* tagging single nucleotide polymorphism, *MAF* minor allele frequency, *CI* confidence interval, *OR* odds ratio

[a] Adjusted for age, gender, APACHE III score, all risk factors for ARDS (listed in Table 1), diabetes, liver cirrhosis/failure and alcohol abuse. APACHE III score was revised to remove age and PaO_2/FiO_2 to avoid colinearity

[b] Regression analysis based on the additive model, indicating the effects of numbers of copies of the minor alleles on ARDS risk

Table 4 Associations between *HMOX1* haplotypes and acute respiratory distress syndrome risk

Haplotypes[a]	Estimated frequency (%)		*P* value	Crude		Adjusted[c]	
	Cases ($n = 437$)	Controls ($n = 1{,}014$)		OR (95% CI)	*P* value	OR (95% CI)	*P* value
Global test[b]				$\chi^2 = 16.91$	0.005	$\chi^2 = 13.98$	0.016
M-AGA	51.2	49.3	0.343	1.16 (0.84–1.60)	0.369	1.18 (0.83–1.66)	0.360
S-TAG	22.5	18.0	0.005*	1.76 (1.19–2.62)	0.005*	1.75 (1.15–2.68)	0.010*
M-TAG	9.8	11.4	0.226	0.71 (0.42–1.20)	0.202	0.60 (0.37–1.06)	0.077
L-AGA	6.3	7.2	0.421	0.78 (0.41–1.50)	0.457	0.74 (0.37–1.50)	0.408
M-TAA	4.3	6.0	0.057	0.51 (0.24–1.09)	0.084	0.59 (0.26–1.33)	0.202

HMOX1 heme oxygenase-1 gene, *OR* odds ratio, *CI* confidence interval, *S* short, *M* middle, *L* long

* FDR adjusted *P* value < 0.05

[a] The polymorphisms in the haplotype are arranged in the order of $(GT)_n$ polymorphism, rs2071746, rs2071748 and rs5755720

[b] Likelihood ratio test, with 5 degrees of freedom

[c] Adjusted for age, gender, APACHE III score, all risk factors for ARDS (listed in Table 1), diabetes, liver cirrhosis/failure and alcohol abuse. APACHE III score was revised to remove age and PaO_2/FiO_2 to avoid colinearity

Discussion

In this study, we found that longer $(GT)_n$ repeats in the *HMOX1* gene promoter were associated with reduced ARDS risk, and the haplotype S-TAG was associated with increased ARDS risk. These associations remained significant after correction for multiple testing. Analysis of association with the intermediate phenotype, plasma HO-1, further supported our findings. Longer $(GT)_n$ repeats were associated with higher plasma HO-1 levels, thus providing protection against ARDS. The tSNPs, however, were not independently associated with plasma HO-1 levels or ARDS risk.

The distribution of $(GT)_n$ repeats in our study population was consistent with previous reports in Caucasians [29–31]. Although this polymorphism has been studied extensively, the standard cutoffs for grouping the $(GT)_n$ repeats remain inconclusive. In this study, we performed a sensitivity analysis to determine the best-fit cutoffs. We also did a thorough review and reclassified the $(GT)_n$ repeats according to the two most commonly used grouping criteria. Our results were robust, consistently showing that longer repeats were associated with reduced ARDS risk. The best-fit cutoffs we found in this study just reflected the two peaks of 23 and 30 repeats. However, this classification needs to be validated in other studies.

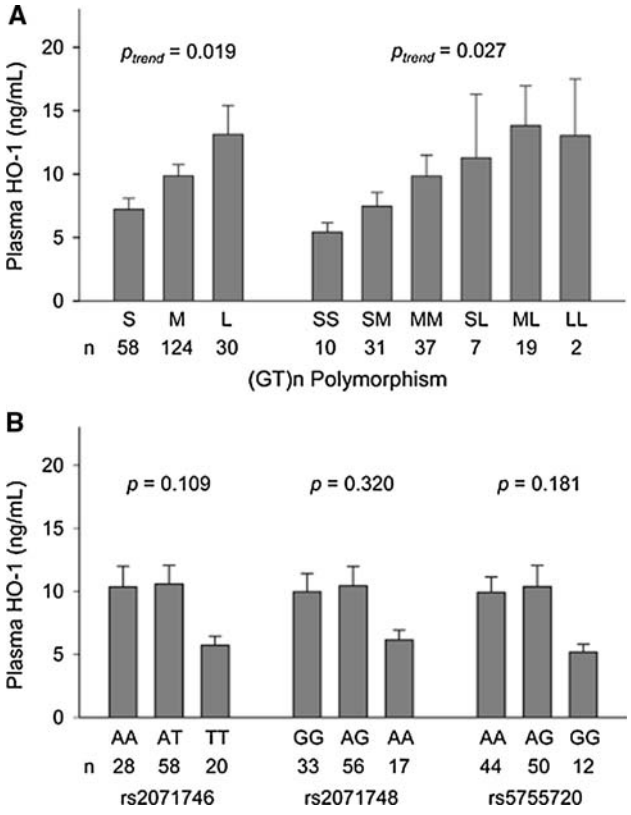

Fig. 2 Plasma heme oxygenase-1 (HO-1) levels among different *HMOX1* genotypes in 106 patients with ARDS. **a** $(GT)_n$ polymorphism. **b** Tagging single nucleotide polymorphisms (tSNPs). Data are expressed in plasma HO-1 levels (ng/mL) and indicate mean + SE of each allele type or genotype. The effects of *HMOX1* genetic variation on plasma HO-1 were tested in linear regression models, in which the dependent variable, plasma HO-1, was Box-Cox transformed ($\lambda = -0.2$) to comply with normality (Kolmogorov–Smirnov equality-of-distributions test, $P > 0.15$). For $(GT)_n$ polymorphism, the P values for trend (P_{trend}) were calculated. For tSNPs, the P values were calculated based on the additive model, indicating the effects of numbers of copies of the minor alleles

Table 5 Regression analysis of correlation between *HMOX1* haplotypes and plasma heme oxygenase-1 levels in 106 patients with acute respiratory distress syndrome

Haplotypes	Coefficient (SE)	P value[a]
M-AGA	−0.022 (0.159)	0.888
S-TAG	−0.299 (0.161)	0.066
M-TAG	0.183 (0.264)	0.489
L-AGA	0.778 (0.252)	0.003*
M-TAA	0.144 (0.356)	0.686

HMOX1 heme oxygenase-1 gene, *SE* standard error, *S* short, *M* middle, *L* long

* FDR adjusted P value < 0.05

[a] Analyzed in a linear regression model, where the dependent variable, plasma HO-1 levels, was transformed to normality by Box-Cox transformation ($\lambda = -0.2$)

Our results showed that longer $(GT)_n$ repeats were associated with higher plasma HO-1 levels and might protect against ARDS. Such functional significance, although being highly internally consistent in this study, is contrary to most previous reports that shorter $(GT)_n$ repeats are associated with higher transcriptional activity, higher HO-1 enzymatic activity, and thus less susceptibility to diseases. Several explanations for the apparent difference are plausible. First, most functional analyses were carried out in vitro by using the transient-transfection assay [16, 19], or by determining the HO-1 expression in specific cell cultures [17–19]. However, a promoter assay in vitro does not necessarily represent the gene expression in vivo. Second, regulation of promoter activity by the length of $(GT)_n$ repeats may differ in cell types in response to stimuli [18, 19], and the influence of a specific allele on the promoter activity may change during inflammatory conditions [32]. Third, many *HMOX1* association studies were conducted in Japanese and Chinese populations. The impact of genetic variation on disease risk may differ among ethnic groups. Noticeably, the presumed protective SS genotype has been associated with higher risk for malignant melanoma [33], cerebral malaria [23], and idiopathic recurrent miscarriage [22]. In addition, some studies showed that the LL genotype, although not statistically significant, may protect against certain diseases [30, 34, 35]. To date, the precise molecular mechanisms by which the $(GT)_n$ repeats modulate the expression of HO-1 remain unknown. Some investigators argue that the alternating purine-pyrimidine sequences have the potential to assume Z-DNA, which exerts a negative effect on transcription [36]. However, longer promoter microsatellite dinucleotide repeats do not necessarily down-regulate the promoter activity (ESM11).

Another possible explanation is that our intermediate phenotype plasma HO-1 protein might not be a good surrogate for lung HO-1 expression, as it could simply reflect the level of tissue damage rather than HO-1 synthesis. Thus the shorter $(GT)_n$ repeats might still be associated with increased tissue HO-1 expression, as would be expected. Excessive induction of HO-1 may be deleterious in critically ill patients [37], possibly through the release of free iron and iron-catalyzed oxidative damage. Studies have shown that changes in plasma and lung iron homeostasis are important in the development and propagation of acute lung injury [38]. Variation in iron homeostasis genes like ferritin light-chain (*FTL*) and heme oxgenase-2 (*HMOX2*) has been associated with ARDS [39]. Our findings further support that these iron-handling genes might participate in the pathogenesis of ARDS.

In addition to the $(GT)_n$ polymorphism, rs2071746 (−413A>T) has also been associated with *HMOX1* promoter activity and is considered dominant over the $(GT)_n$ repeats [31, 40]. Our data, however, indicate a functional

dominance of the $(GT)_n$ repeats over rs2071746 (see ESM12 for the detailed discussion).

A major strength of this study is that it was conducted within a large, well-defined, prospectively enrolled cohort of patients at risk for ARDS. The restriction of our analysis to Caucasians and the use of at-risk patients as controls also minimized the possible confounding from either ethnicity or the genetic associations with predisposing conditions of ARDS like sepsis or pneumonia. To our knowledge, this is the first study evaluating both $(GT)_n$ polymorphism and tSNPs in *HMOX1*. We reported the LD between $(GT)_n$ polymorphism and tSNPs for the first time. In addition, we also measured plasma HO-1 as an intermediate phenotype to analyze the functional significance of *HMOX1* polymorphisms in ARDS patients. Our results were very consistent. The findings from genotype and haplotype analyses were consonant with each other, and the genotype–phenotype and genotype-disease associations were in a concordant direction.

One of the limitations in this study is that we measured plasma HO-1 in a mere subset of ARDS patients due to availability of acute-phase plasma samples. Although the genotype distributions were not different between patients with and without plasma samples tested, there might remain selection bias in the association of *HMOX-1* variants with plasma HO-1. Moreover, we were not able to assess the association between plasma HO-1 levels and ARDS development because this plasma subset was a case-only subgroup. Another concern is the correspondence between plasma and tissue (lung) HO-1 expressions in response to stress, which need to be investigated. Finally, our results were based on a single population, thus need to be validated in other populations.

Conclusion

Longer $(GT)_n$ repeats in the *HMOX1* promoter are associated with higher plasma HO-1 levels and reduced ARDS risk. The common haplotype S-TAG is associated with increased ARDS risk. Nevertheless, no individual tSNP is associated with plasma HO-1 levels or ARDS risk. Our results suggest that *HMOX1* variation may modulate plasma HO-1 levels and ARDS risk through the promoter microsatellite polymorphism. Further studies are needed to confirm our findings. The standard classification of $(GT)_n$ repeats and the correspondence of HO-1 levels in plasma and tissues also need to be investigated.

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Conflict of interest statement The authors have not disclosed any potential conflicts of interest.

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