4G/5G Plasminogen Activator Inhibitor-1 Polymorphisms and Haplotypes are Associated with Pneumonia

ONLINE DATA SUPPLEMENT

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METHODS

Population

The Health ABC Study is a prospective, community-based study designed to investigate the effects of body composition on morbidity, functional limitations, and mortality. A total of 3,075 participants were recruited in 1997 and 1998 from a random sample of white and black Medicare beneficiaries residing within ZIP codes from the metropolitan areas in and surrounding Pittsburgh, Pennsylvania and Memphis, Tennessee. Eligibility criteria were; 70-79 years of age, reported no difficulty walking quarter mile, climbing up ten steps, and performing mobility-related activities of daily living, no radiation treatment or chemotherapy for cancer in the past three years, not enrolled in a trial of a lifestyle intervention, and had no plans to move out of the area in the next three years.

Outcome measure

The primary outcome was CAP requiring hospitalization. During the seven years of follow-up in the Health ABC cohort hospitalization was ascertained based on participant self-report and active surveillance by study personnel. Adjudication of CAP was performed by an adjudicator at each site and criteria to adjudicate CAP were established prospectively. We used a combination of discharge summary, ICD-9 diagnoses, admission history and physical examination, and radiology reports for each hospitalization to ascertain CAP requiring hospitalization. The Health ABC study also utilized Health Care Financing Administration (HCFA) data for hospitalization in the preceding five years based on ICD-9 discharge diagnosis. The following ICD-9 codes were used to diagnose CAP: 481 (pneumococcal pneumonia), 482.0 (pneumonia due to Klebsiella pneumoniae), 482.1 (pneumonia due to Pseudomonas), 482.2

(pneumonia due to Hemophilus influenzae), 482.3 (pneumonia due to Streptococcus), 482.4 (pneumonia due to Staphylococcus), 482.83 (pneumonia due to other gram negatives), 482.9 (bacterial pneumonia, unspecified), 483.0 (pneumonia due to Mycoplasma pneumoniae), and 486 (pneumonia, organism unspecified).

PAI-1 gene

The MassARRAY system (Sequenom Inc., San Diego, CA), a mass spectroscopy-based high-throughput single nucleotide polymorphism genotyping system, was employed. Each DNA plate contained two positive controls (CEPH DNA from individuals 1331-01 and 1331-02) at different locations. For each polymorphism genotyped, the genotype of each CEPH control was confirmed. Additionally, two negative controls (i.e., water only) were included on each plate to identify potential contamination during the polymerase chain reaction setup. Repeated genotyping was conducted for 10% of samples in a masked fashion. The kappa statistic for reliability was 1.0.

European ancestry markers

We genotyped 37 ancestry-informative genetic markers, which are known to differ in allele frequency by 0.6 or more (mean difference = 0.79) between European and African populations (1). These markers occur at spacing of at least 20 centimorgans across chromosomes 1 to 22, and are not in linkage disequilibrium in either parent population. Using these genotype data, we estimated the proportion of European ancestry for each self-reported black participant in Health ABC by employing a maximum likelihood estimation procedure. This methodology has been shown to give estimates of European ancestry that correspond with high fidelity to values obtained from a Markov Chain Monte Carlo approach (2).

Cytokine concentrations

Blood samples were collected by venipuncture after an overnight fast at mean time 0925 hours in the absence of infection at enrollment into the Health ABC study. The specimens were aliquoted into cryovials, frozen at –70°C, and shipped to the Health ABC Core Laboratory at the University of Vermont. The PAI-1 level was measured from citrated plasma with a two-site ELISA (Center of Molecular and Vascular Biology, University of Leuven, Belgium). The assay is sensitive to free PAI-1 (both latent and active) but not PAI-1 in complex with tissue plasminogen activator. Plasma TNF and IL-6 levels were measured in duplicate by ELISA kits (R&D Systems, Minneapolis, MN) using HS600 Quantikine and HSTA50 kits, respectively. Reliability was ascertained using a blind duplicate system and the inter-assay co-efficient of variation (CV) for the PAI-1 assay was 3.5%.

Whole blood *ex vivo* stimulation with lipopolysaccharide (LPS) and peptidoglycan (PGN)

To evaluate functional significance of PAI-1 polymorphisms, we compared PAI-1, TNF, IL-6, and IL-10 concentrations for different genotypes following *ex vivo* whole blood stimulation with LPS and PGN. Briefly, 23 healthy volunteers without chronic health conditions at the Pittsburgh site were recruited. All participants gave informed consent and the institutional review board at the University of Pittsburgh approved the study. Blood was collected with aseptic precautions in heparinized tubes and transported to the laboratory immediately at room temperature. Upon arrival in the lab, blood was incubated for 24 hours with LPS (500ng/ml) from Salmonella Minnesota (Calbiochem Inc., San Diego, CA) and PGN (100µg/ml) from Staphylococcus aureus (Fluka, Sigma Aldrich Inc., St Louis, MO) in six well plates. For each subject, a negative control using similar experimental conditions but without LPS and PGN was performed. Appropriate concentrations of LPS and PGN were prepared after dilution with

RPMI-1640 medium (Sigma Aldrich Inc., St Louis, MO) and endotoxin-free water (Sigma Aldrich., St Louis, MO), whereas RPMI-1640 medium was used as negative control. To choose optimal concentration of LPS and PGN and duration of incubation for this experiment, we examined cytokine response to whole blood stimulation in a single subject using different incubation periods (4, 24, 48, and 72 hours) and using different concentrations of LPS and PGN (data not shown). Cytokine concentrations in the supernatant were measured at 24 hours in LPS stimulated, PGN stimulated, and control wells. PAI-1 was measured by an ELISA kit (Quantikine assay, R & D systems, Minneapolis, MN). TNF, IL-6, and IL-10 were measured using chemiluminescent immunoassay using an automated analyzer (IMMULITE, Diagnostic Products Corp., Los Angeles, CA).

Covariates

At the baseline visit, age, gender, and race were assessed along with a detailed health history and medication inventory. Prevalent health conditions, such as congestive heart failure, coronary heart disease, diabetes, and smoking were determined by a combination of self-report and use of specific medications, whereas serum creatinine was used to ascertain renal function.

Pulmonary function test

Spirometry was performed approximately two weeks after enrollment during clinic visit with a horizontal dry rolling seal spirometer to ascertain lung function. Forced vital capacity (FVC) and forced expiratory volume in first-second (FEV_1) had to meet American thoracic society (ATS) criteria for acceptability and reproducibility (3, 4).

Statistical analyses

Univariate analysis was performed using chi-square, t-test, and Wilcoxon test, as appropriate, to compare frequency of CAP and to compare PAI-1 levels. Association between individual SNP's and CAP frequency was assessed using chi-square test for genotype and allele. Logistic regression models were used for multivariable analysis and model fit was ascertained by Hosmer-Lemeshow test.

We constructed haplotypes using PROC HAPLOTYPE option in SAS Genetics (version 9.1, SAS Institute Inc., Cary, NC). We used haplotype marker trait association to test association between haplotypes and CAP susceptibility. Since haplotypes cannot be assigned unequivocally for heterozygote genotypes, we estimated probability of individual haplotype used haplotype trend analysis. These probability scores were included in the logistic regression analysis to assess if the association between haplotypes and CAP susceptibility is independent of covariates.

For whole blood stimulation assays, we compared difference in cytokine concentrations following LPS or PGN stimulation and control conditions for different genotypes. We log converted TNF, IL-6, and IL-10 concentrations because they did not follow normal distribution. Student's t-test was used to compare cytokine concentrations for different genotypes.

REFERENCES

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E2. Pritchard JK, Stephens M, Donnelly P. Inference of Population Structure Using Multilocus Genotype Data. *Genetics* 2000;155:945-959.

E3. Lung function testing: selection of reference values and interpretative strategies. American Thoracic Society. *Am Rev Respir Dis* 1991;144:1202-1218.

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Table E1. -- Frequency of common single nucleotide polymorphisms within the PAI-1 gene (minor allele frequency>10%) available at the Seattle SNPs website. Each set of rows represents a single haplotypes bin. Only polymorphisms with minor allele frequency >10% are shown.

SNPs from the Seattle SNPs website		Minor allele frequency in Blacks	Minor allele frequency in Whites
SNPs included in our study	Other SNPs from the same haplotype bin		
837		0.33	0.84*
	664	0.37	0.92*
4588		0.12	0.29
	5686	0.12	0.31
	5404	0.15	0.35
7343		0.55	0.31
	13605	0.33	0.28
	5878	0.38	0.28
12219		0.83	0.50
	5645	0.28	0.48
	7121	0.92	0.52
	7437	0.92	0.48
	8070	0.92	0.47
	8406	0.92	0.48
	9463	0.92	0.47
	9466	0.92	0.47
	12580	0.84	0.48
	13889	0.85	0.47
12750		0.05	0.28

*Frequency of variant allele varied by race. Therefore, minor allele frequency exceeds 50%

Table E2. -- Linkage disequilibrium* between single nucleotide polymorphisms stratified by race.

	PAI4G,5G rs799889	**PAI2846** rs6092	**PAI2852** rs6090	**PAI4588** rs2227657	**PAI7343** rs2227674	**PAI12219** rs11178	**PAI12750** rs1050813
Whites							
PAI837		0.46 (0.37)	0.47 (0.13)	0.01 (0.01)	0.47 (0.53)	0.45 (0.74)	0.31 (0.34)
PAI2846			0.58 (0.05)	0.49 (0.02)	0.48 (0.09)	0.47 (0.31)	0.57 (0.21)
PAI2852				0.48 (0.29)	0.42 (0.05)	0.45 (0.1)	0.48 (0.06)
PAI4588					0.56 (0.23)	0.52 (0.36)	0.45 (0.17)
PAI7343						0.48 (0.45)	0.56 (0.3)
PAI12219							0.49 (0.45)
Blacks							
PAI837		0.46 (0.09)	0.56 (0.18)	0.19 (0.09)	0.25 (0.19)	0.26 (0.34)	0.42 (0.11)
PAI2846			0.23 (0.02)	0.05 (0.01)	0.46 (0.23)	0.63 (0.18)	0.59 (0.04)
PAI2852				0.51 (0.67)	0.53 (0.17)	0.48 (0.23)	0.85 (0.1)
PAI4588					0.46 (0.22)	0.56 (0.41)	0.52 (0.09)
PAI7343						0.46 (0.55)	0.31 (0.08)
PAI12219							0.61 (0.26)

*Linkage disequilibrium estimated using Lewontin's D′ and correlation co-efficient (in parenthesis)

Table E3. -- Association between CAP requiring hospitalization and haplotypes based on 4 SNPs among white participants after excluding CAP events prior to enrollment*

Haplotypes					Frequency of haplotypes			P value
PAI 4G,5G	PAI 2846	PAI 4588	PAI 7343	PAI 12750	Overall	CAP participants	Participants without CAP	
5G	A	C	G	-	10.7	5.4	11.1	0.006
5G	G	C	A	-	18.6	19	18.9	0.93
5G	G	C	G	-	9.7	10	9.7	0.85
5G	G	T	A	-	6.3	7.9	6.1	0.35
4G	G	C	A	-	46.4	52.9	45.9	0.06
4G	G	T	A	-	6.6	5.2	6.7	0.49

*P=0.059 for overall marker-trait association

Table E4. -- Lack of association between PAI 4G,5G genotypes with age and mortality in white participants

Variable	4G/4G genotype (%)	4G/5G genotype (%)	5G/5G genotype (%)	P value
Age				
<72 years	32	47	21	0.6
72-74 years	30	49	21	
75-77 years	27	50	23	
≥78 years	30	47	23	
Mortality				
Participants with CAP*	30	31	25	0.9
Participants without CAP*	14	14	14	0.9

*Community acquired pneumonia