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ARTICLE

Genetic variation at the human tissue-type plasminogen activator (tPA) locus: haplotypes and analysis of association to plasma levels of tPA

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Tissue-type plasminogen activator (tPA) plays a key role in thrombus dissolution and plasma levels of tPA have been associated with cardiovascular disease. We have previously resequenced regulatory and coding regions of the human tPA gene (PLAT) and identified eight single-nucleotide polymorphisms (SNPs). In a small experimental study, four common variants were associated with invasively determined vascular tPA release rates. The aim of the present study was to investigate whether there is an association between genetic variants at this locus and plasma levels of tPA. To this end, 240 Swedish individuals without cardiovascular disease were typed for the eight SNPs and an Alu insertion polymorphism at the PLAT locus, as well as for a polymorphism in the plasminogen activator inhibitor type 1 (PAI-1) promoter (PAI-1 −675 4G > 5G). Stepwise regression analysis, with established predictors of plasma tPA including plasma PAI-1 and genetic variants, showed that neither genotypes nor haplotypes were major contributors to plasma tPA. The results also showed that the level of linkage disequilibrium was high at the PLAT locus, as demonstrated by the fact that only three haplotypes had a frequency above 5%. In conclusion, in the present study neither genetic variation at the PLAT locus nor the PAI-1 −675 4G > 5G polymorphism was strong predictors of plasma tPA levels, which suggests that variations in other genes contribute to the heritability of this phenotype. The results also show that three haplotypes at the PLAT locus accounted for nearly 90% of the chromosomes and that they could be defined by typing only two SNPs.
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Introduction

Tissue-type plasminogen activator (tPA) is a serine protease that catalyzes the conversion of the zymogen plasminogen to the active enzyme plasmin. Plasmin, in turn degrades fibrin, which forms the matrix structure of a blood clot. Thus, tPA plays a key role in thrombus dissolution.[1,2] Studies on tPA-deficient mice have confirmed the importance of tPA in mediating thrombolysis[3,4] and plasma levels of tPA have been associated with myocardial infarction and stroke.[5–7]

tPA is synthesized in endothelial cells and its gene expression is mainly regulated at the level of transcription.[8] The human gene encoding tPA, PLAT, is located on chromosome eight. It spans >30 kb and consists of 14 exons.[9] The promoter contains two transcription start sites,

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which are dependent on an initiation element and a TATA box, respectively.[9,10] Two elements have been identified within the proximal promoter that are responsible for both constitutive expression and induction by the protein kinase C pathway.[11] In addition, a multihormone-responsive enhancer located −7.3 kb from the initiator element has been identified.[12] In a previous study, we resequenced. the enhancer, the proximal promoter, all 14 exons and their flanking intronic sequences, and the immediate 3′ region of PLAT.[13] We identified eight novel single-nucleotide polymorphisms (SNPs): one in the enhancer (−7,351C>T), one just upstream, and one just downstream of the initiator element (−125T>C and 90A>G), three in coding sequence (two synonomous; 20,099T>C and 28,792T>C and one nonsynonomous; Arg129Trp), and two in intronic sequence (27,445T>A and 27,739G>A). In that experimental study, we also found an association between four of the SNPs and invasively determined vascular release rates of tPA.[13] The closest association was observed for tPA −7,351C>T SNP. Subjects homozygous for the tPA −7,351C allele had more than two-fold higher tPA release rates as compared to carriers of the T allele. In contrast, although the CC group tended to have higher plasma levels of tPA antigen, there was no significant association between the −7,351C>T SNP and plasma tPA.

In contrast to vascular tPA release, plasma tPA is not only dependent on endothelial release, but also on hepatic clearance and complex inhibition.[13,14] Our recent data from *in vitro* studies show that the tPA −7,351C>T SNP is functional at the level of transcription [Ladenvall *et al*[13] and unpublished observations]. It is therefore conceivable that the phenotype vascular tPA release, which is highly dependent on the synthesis rate, more closely reflects tPA −7,351C>T genotype than plasma tPA. It follows that a larger sample size would be needed to detect a possible association between this SNP and plasma tPA. Consequently, the present study was designed to investigate whether the tPA −7,351C>T SNP is associated with plasma tPA or not, and data from our initial study were used to estimate the required sample size. To fully assess whether genetic variation at the PLAT locus contributes to plasma tPA, all nine polymorphisms as well as haplotypes were analyzed, and established predictors of plasma tPA as well as a polymorphism in the plasminogen activator inhibitor type 1 (PAI-1) promoter were also taken into account.

Materials and methods

Study population

In the present study, we re-examined 240 unrelated men and women from the population-based Göteborg MONICA study.[15] Subjects with a history of stroke, coronary or peripheral artery disease were excluded. Out of 415 eligible subjects, 81 did not respond, 44 subjects were unwilling to attend without any particular reason, 25 subjects were unwilling to attend because of illness. Six of the subjects had moved out of the region. In all, 11 subjects were excluded at the examination and five subjects after the examination because of history or ECG findings indicating cardiovascular disease. Three subjects were excluded for technical reasons. This left 240 subjects from western Sweden without cardiovascular disease which constitute the current study. All subjects were white, 88% were native Swedes and 61% of these were born in Göteborg or western Sweden. The study was approved by the Ethics Committee of Göteborg University, and the data handling procedures were approved by the National Computer Data Inspection Board. All participants gave their written informed consent.

Clinical characteristics

Subjects were examined between 08.00 and 10.00 h after an overnight fast. The participants were asked to complete a questionnaire with, *inter alia*, items on smoking habits and history of cardiovascular disease. A 12-lead ECG was recorded and evaluated by the Minnesota code.[16] Body mass index (BMI) was calculated from weight in kilograms divided by height squared in meters. Blood pressure was measured in the supine position after 10 min rest. Cuff size was adjusted for arm circumference. Subjects fulfilling at least one of the following criteria were defined as hypertensives: regular treatment with antihypertensive drugs at the time of examination, a systolic blood pressure ⩾160 mm Hg, or a diastolic blood pressure ⩾95 mmHg. Individuals who had a fasting plasma glucose ⩾7.0 mmol/l or were treated with insulin, oral antidiabetic drugs or on diet control at the time of examination were defined as diabetics. Smoking history was classified into subjects who were current smokers and non-smokers (exsmokers and people who never smoked).

Blood sampling

Blood samples were drawn from an anticubital vein, with subjects in the supine position, after 20 min rest. Samples for determination of fibrinolytic variables were collected in tubes containing 1/10 0.45 M sodium citrate buffer, pH 4.3 (Biopool Stabilyte™, Biopool International, Umeå, Sweden). Blood collection tubes were kept on ice and plasma was isolated within 30 min by centrifugation at 2000 *g* for 20 min at 4°C. Plasma aliquots were immediately frozen and stored at −70°C until analysis.

Biochemical assays

Hematocrit, plasma glucose, and serum concentrations of cholesterol, high-density lipoprotein (HDL), and triglycerides were determined by standard methods at the Department of Clinical Chemistry, Sahlgrenska University Hospital.

Plasma concentrations of tPA antigen were determined with an enzyme-linked immunosorbent assays (ELISA),

which detects free and complexed forms of tPA with similar efficiency (TintElize® tPA, Biopool International).[17] Plasma tPA activity, that is, the free, uncomplexed fraction of tPA, was measured by a bioimmunoassay (Chromolize™ tPA, Biopool International) calibrated against the international standard for tPA (lot 86/670). Active tPA was expressed in μg/L using the specific activity of 600 IU/μg (data on file, Biopool International). Plasma levels of PAI-1 antigen were determined by an ELISA, which detects all molecular forms of PAI-1 with similar efficiency (COALIZA® PAI-1, Haemochrom Diagnostica, Mölndal, Sweden).[18] All samples were assayed in duplicate on the same microtiter plate. Mean intra-assay coefficients of variation (CV) were 2.6, 3.0, and 3.4% for tPA antigen, tPA activity, and PAI-1 antigen, respectively. The corresponding interassay CVs were 6.3, 6.3, and 14.8%. Intraindividual reproducibility of fasting morning plasma levels of tPA and PAI-1 antigen has previously been reported to be high in healthy people.[19]

The tPA Arg129Trp SNP causes an amino-acid substitution in kringle one, which theoretically might affect the affinity of antibodies for tPA. However, since both antibodies in the TintElize® tPA kit are polyclonal, it is unlikely that the amino acid replacement affects the quantification of tPA antigen. In contrast, the capture antibody in the Chromolize™ tPA kit is monoclonal. It interferes with fibrin binding and is thus most likely directed towards the finger domain or kringle two. However, since it cannot be excluded that the epitope is located in kringle one, 12 samples from subjects carrying the Trp129 allele and 12 samples from subjects homozygous for the Arg129 allele were analyzed by a functional assay without the use of antibodies (Spectrolyse®/fibrin, Biopool International). Samples from the two groups were matched for plasma tPA activity as determined by Chromolize™ and mean tPA activity was 1.02 (range 0.58–1.67) μg/l in both groups. There was no significant difference in tPA activity as determined by Spectrolyse® between the two groups; mean 1.13 (range 0.65 – 1.60) and mean 1.10 (range 0.68 – 1.47) μg/l in the Arg129/Trp129 and Arg129/Arg129 group, respectively. These results indicate that the amino acid substitution does not affect the affinity of the monoclonal antibody.

Genotyping

Genomic DNA was isolated from whole blood on Qiagen columns (QiaAmp, Qiagen, Hilden, Germany). Genotyping of SNPs were performed by 5′ nuclease (TaqMan) assays. The principle of this assay is that the polymorphic region is amplified with PCR in the presence of two competing probes, each complementary to one of the SNP alleles.[20] Oligonucleotide primers and probes were designed from the DNA sequences obtained in our earlier work[13] using Primer Express 1.5 software (Applied Biosystems Inc, Foster City, CA, USA). Primers were designed to amplify short target DNA fragments (<170 bp) with the SNP of interest. Probes representing each allele were designed. Probes complementary to the common alleles were labeled at the 5′ end with VIC and probes complementary to the rare alleles were labeled with FAM (6-carboxy-flourescein). Both probes also had a quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) on the 3′ nucleotide and probe lengths were adjusted to a T_m of 65–67°C. Ordinary TaqMan® probes were used. However, if the polymorphic site was located in an AT-rich region, probes with a minor groove binder (MGB) modification were used, which enabled us to reduce the length of the probe. MGB probes were also chosen if probe design was restricted by neighboring repetitive sequences or SNPs. Primer and probe sequences for tPA SNPs are listed in Table 1. All oligonucleotides were synthesized by Applied Biosystems.

PCR and fluorescence readings were performed in 96-well microtiter plates (MicroAmp® Optical plates, Applied Biosystems) on an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). Reactions (25 μl total volume) consisted of 20 ng genomic DNA, 12.5 μl TaqMan® Universal PCR Master Mix (Applied Biosystems), and primers and probes as indicated in Table 1. Primer concentrations were optimized by amplifying DNA from a subject homozygous for the common allele in the presence of the corresponding probe. Concentrations of the two probes were optimized to give similar fluorescent signals. Thermal cycling conditions were: two initial holds (50°C for 2 min and 95°C for 10 min) followed by a 40-cycle two-step PCR (95°C for 15 s and 58°C or 60°C for 1 min). The fluorescent signals of the two reporter dyes were determined directly after PCR. Each 96-well PCR plate included six samples without DNA as negative nontemplate controls (NTC) and duplicate samples of the three genotypes as positive controls.

To validate each 5′ nuclease assay, 51 samples that had been genotyped earlier by DNA sequencing[13] were run. All genotypes obtained with the 5′ nuclease assays were concordant with DNA sequencing. In addition, 10% of the samples in the present study underwent repeat genotyping by the 5′ nuclease assays and the initial results were confirmed in all cases.

Genotyping of the Alu insertion polymorphism was performed by PCR as previously described.[21] To avoid misclassification of DD genotypes, all samples genotyped as DD were amplified by a second PCR with an insertion-specific primer.[21]

Genotyping of the PAI-1 −675 4G>5G polymorphism was performed by a 5′ nuclease assay as previously described, and the accuracy of this assay has been verified by direct DNA sequencing.[22]

Statistical analysis

Allele frequencies were estimated from genotype data. Deviation from Hardy–Weinberg equilibrium was tested by a χ^2 test with one degree of freedom. Pairwise linkage

Table 1 5′ nuclease assays for SNPs at the PLAT locus

Oligonucleotide	*Concentration (nM)*
tPA –7,351C >T	
FP: 5′-AGTGATCTCATTGCCGAGGTG-3′	300
RP: 5′-CCCAGAGTCCCAGGCCA-3′	300
C-TQMP: 5′-AAAGGAGCC**C**GCCCCAGACA-3′	100
T-TQMP: 5′-CCAAAGGAGCC**T**GCCCCAGAC-3′	100
tPA –125T >C	
FP: 5′-TGCTTCCACCGTGAACTTCC-3′	300
RP: 5′-TCAGTGGACAGGGCCATGA-3′	300
T-TQMP: 5′-AACAGGCC**T**GCCTCAGCTCCC-3′	100
C-TQMP: 5′-ACAGGCC**C**GCCTCAGCTCC-3′	100
tPA 90A >G	
FP: 5′-GCTTGCTGGAAAGACTTTTAAGAGA -3′	300
RP: 5′-CCTACAGGAGTCCAGGGCTG-3′	300
A-TQMP: 5′-AGACCCCAAGG**T**ACAGAAACCCGA-3′	100
G-TQMP: 5′-AGACCCCAAGG**C**ACAGAAACCC-3′	100
tPA Arg129Trp	
FP: 5′-GACAGAATCTTTCCCCAGCTGTA-3′	300
RP: 5′-GTTGGCCCAGAAGCCCTAC-3′	300
C-MGBP-2: 5′-TCTGGCCTCC**G**CCC-3′	50
T-MGBP: 5′-CTGGCCTCC**A**CCC-3′	200
tPA 20,099T >C	
FP: 5′-GACAGAATCTTTCCCCAGCTGTA-3′	900
RP: 5′-GTTGGCCCAGAAGCCCTAC-3′	900
T-MGBP-2: 5′-GATGGC**A**TCTGGCCT-3′	50
C-MGBP-2: 5′-ATGGC**G**TCTGGCC-3′	200
tPA 27,445T >A	
FP: 5′-TCCACCCTCTAACGCCTAGAAAC-3′	900
RP: 5′-CCGGTATGTTCTGCCCAAGA-3′	900
T-MGBP: 5′-CACAGCAAAAA**T**AGCAT-3′	100
A-MGBP: 5′-AGCAAAAA**A**AGCATTCT-3′	200
tPA 27,739G >A	
FP: 5′-CATTGGTAAGAGCTCGTCATTCC-3′	300
RP: 5′-GAGGAGTGAGCTGGCGTGA-3′	300
G-TQMP: 5′-TCCCG**G**GGACGCGGC-3′	200
A-TQMP: 5′-CTCCCG**A**GGACGCGGCC-3′	100
tPA 28,792T >C	
FP: 5′-TCCCTAAAACCATATGATGGTTCTC-3′	300
RP: 5′-GCCTCCTTCAGCCGCTC-3′	900
T-MGBP: 5′-TTCAGTGTC**T**CCTTTCT-3′	100
C-MGBP: 5′-TCAGTGTC**C**CCTTTC-3′	200

TQMP, TaqMan probe; MGBP, minor grove binder probe; FP, forward primer; RP, reverse primer. Annealing temperatures were 58°C for tPA Arg129Trp and tPA 20,099T>C and 60°C for the other SNPs.

disequilibrium (LD) coefficients, D', were calculated[23] and LD was tested by a likelihood ratio χ^2 test. Haplotype frequencies were estimated by the expectation-maximization (EM) algorithm as implemented in the EH software.[24] Since fibrinolytic variables, triglycerides and HDL were positively skewed, these variables were transformed by the natural logarithm and all subsequent analyses were performed on transformed values. Correlations between established predictors of plasma tPA and plasma levels of tPA antigen and activity were assessed by Pearson's correlation coefficient. Associations between tPA and PAI-1 genotypes and plasma tPA were tested by stepwise linear regression analysis using the SPSS 10.0 for Macintosh package. All 10 polymorphisms, as well as established predictors of plasma tPA, were allowed to enter each model. Sex, smoking, hypertension, and diabetes were treated as categorical variables, and age, BMI, cholesterol, HDL, triglycerides, hematocrit, PAI-1 antigen, tPA antigen, and tPA activity were treated as continuous variables. F-to-enter was set to 3.84 (corresponding to $P<0.05$). Similar stepwise models were applied to test association between common haplotypes and plasma tPA. By bootstrapping 10,000 times from the data of our initial study[13] the power of the present study to detect an association between the tPA −7,351C>T SNP and plasma tPA antigen concentrations was estimated to 90%.

Missing values

The number of individuals with missing values were; two for smoking status and one for BMI, systolic blood pressure, diastolic blood pressure, glucose, and hematocrit.

Results

Baseline characteristics of study population

Baseline characteristics of the 240 subjects as well as plasma levels of tPA and PAI-1 are shown in Table 2. Of the 47 subjects who were classified as hypertensives, 22 were on regular treatment with antihypertensive drugs and 25 had a high blood pressure at examination. Eight subjects were classified as diabetics of whom two were on regular treatment with insulin, two on oral antidiabetic drugs, three on diet control, and one subject had an elevated plasma glucose level at examination.

Allele frequencies, LD, and estimation of haplotypes

Allele frequencies for the tPA polymorphisms are presented in Table 3. The frequency for the PAI-1 4G and 5G allele was 0.52 and 0.48, respectively. There were no significant deviations for the observed genotype distributions from those predicted with the assumption of Hardy–Weinberg equilibrium for any of the studied polymorphisms.

LD was estimated for each pair of sites at the PLAT locus using the measure of D'. All polymorphisms were in high and significant LD with each other ($D' > 0.6$ and $P<0.01$), except for the most 5′ site versus the two most 3′ sites, as well as the rare Arg129Trp variant *vs* 27,445 (Table 3). A total of 24 haplotypes, which represent 4.6% of the 512 possible haplotypes, were inferred from the 480 chromosomes by the EM algorithm. Three haplotypes occurred with a frequency >5%, and they alone accounted for 88% of the 480 chromosomes (Table 4). Six haplotypes

Table 2 Characteristics of subjects

Variable	*Mean (SD)*
Age (years)	54 (12)
Male (%)	59
Current smokers (%)	24
Hypertension (%)	20
Diabetes (%)	3
SBP (mmHg)	131 (20)
DBP (mmHg)	81 (10)
BMI (kg/m^2)	26 (4)
Cholesterol (mmol/l)	5.2 (1)
Triglycerides (mmol/l)	1.2 (0.6)
HDL (mmol/l)	1.4 (0.4)
Hematocrit (%)	42 (4)
tPA antigen (μg/l)	9.0 (1.5)
tPA activity (μg/l)	0.44 (1.9)
PAI-1 antigen (μg/l)	32.9 (1.9)

n=240. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1. For skewed variables, geometric means and geometric standard deviations are shown.

(1.2% of possible haplotypes) occurred with a frequency >1%, accounting for 94% of all chromosomes. Haplotypes were also assigned to each individual, using the most probable pair of haplotypes according to the inferred haplotype frequencies. Since 105 individuals were homozygous for all nine sites or heterozygous at only one site, 210 chromosomes could be unambiguously haplotyped. Frequencies of the individually assigned haplotypes were very similar to frequencies derived from group data (data not shown).

Predictors of plasma tPA levels

Correlations between plasma tPA and established predictors are shown in Table 5. There were significant positive correlations between plasma tPA antigen and age, blood pressure, BMI, cholesterol, triglycerides, hematocrit, and PAI-1, whereas a negative correlation was observed for HDL. Plasma tPA activity was negatively correlated with BMI, triglycerides, and PAI-1 and positively with HDL.

Association between tPA genotype and plasma tPA

Both additive and dominant (for the minor allele) models were tested together with established predictors of tPA. A recessive model was not tested, since there were few individuals homozygous for the minor allele in the present sample. All ten polymorphisms were tested as predictors of plasma tPA in a stepwise model together with age, sex, BMI, cholesterol, HDL, triglycerides, PAI-1, hematocrit, hypertension, diabetes, and smoking. When applying this model with the assumption of an additive effect of alleles,

Table 3 Allele frequencies and pairwise LD coefficients between polymorphisms at the tPA locus

			D′								
Polymorphism	*Allele*	*Allele frequencies*	*−7,351*	*−125*	*90*	*Codon 129*	*20,099*	*Alu*	*27,445*	*27,739*	*28,792*
–7,351	C>T	0.70/0.30		0.62	1	1	0.93	0.89	0.81	0.07	0.05
−125	T>C	0.95/0.05			1	1	1	0.75	−1	0.70	0.70
90	A>G	0.96/0.04				1	1	0.70	−1	1	1
Codon 129	Arg>Trp	0.98/0.02					1	0.79	−0.99	1	1
20,099	T>C	0.58/0.42						0.97	0.95	1	0.97
Alu	I>D	0.59/0.41							0.95	0.93	0.93
27,445	T>A	0.73/0.27								−1	−1
27,739	G>A	0.86/0.14									1
28,792	T>C	0.86/0.14									

D′ were significantly different from zero ($P<0.01$) for all pairwise combinations except for tPA -7,351 in relation to tPA 27,739 and 28,792 (P>0.5 for both) and tPA Arg129Trp in relation to PA 27,445 (P=0.15).

Table 4 Estimated haplotypes at the human tPA locus

Haplotype	*−7,351*	*−125*	*90*	*Codon 129*	*20,099*	*Alu*	*27,445*	*27,739*	*28,792*	*Estimated frequency*
H1	C	T	A	Arg	T	I	T	G	T	55.8
H2	T	T	A	Arg	C	D	A	G	T	23.3
H3	C	T	A	Arg	C	D	T	A	C	8.7
H4	C	T	A	Arg	C	D	A	G	T	2.3
H5	T	C	G	Trp	C	D	T	A	C	2.3
H6	T	C	G	Arg	C	D	T	A	C	1.5

Three haplotypes accounted for 88% of the chromosomes in the current sample. Only haplotypes with an estimated frequency >1% are shown. The remaing 18 estimated haplotypes accounted for 6.1% of the chromosomes.

Table 5 Correlation coefficients between plasma levels of tPA and age, blood pressure, metabolic variables, PAI-1, and hematocrit

	tPA antigen	*tPA activity*
Age	0.41**	0.12
Systolic blood pressure	0.32**	0.01
Diastolic blood pressure	0.33**	−0.11
Body mass index	0.41**	−0.35**
Cholesterol	0.22**	−0.04
Triglycerides	0.44**	−0.26**
High-density lipoprotein	−0.28**	0.26**
Plasminogen activator inhibitor type 1	0.65**	−0.66**
Hematocrit	0.44**	−0.09

Correlation coefficients for skewed variables were performed after logarithmic transformation. **$P<0.01$ (two-tailed).

old age ($P<0.001$), male sex ($P<0.05$), high levels of triglycerides ($P<0.001$), PAI-1 ($P<0.001$), hematocrit ($P<0.001$), and the tPA 90A allele ($P<0.05$) emerged as independent predictors of high tPA antigen ($R^2=0.66$). When applying a dominant model (subjects homozygous for the major allele *vs* carriers of the minor allele at each polymorphic site), the same predictors emerged except for tPA 90A/G ($R^2=0.66$). Similar stepwise models were applied for tPA activity. In the additive model, young age ($P<0.001$), low PAI-1 antigen ($P<0.001$), and the tPA Trp129 allele ($P<0.05$) were identified as independent predictors of high tPA activity ($R^2=0.49$). In the model with the assumption of a dominant effect of the minor allele, young age ($P<0.001$), high levels of HDL ($P<0.05$), low PAI-1 antigen ($P<0.001$), and the tPA 90G allele ($P<0.01$) emerged as significant predictors of high tPA activity ($R^2=0.50$).

Association between tPA haplotype and plasma tPA

The three most common haplotypes with a frequency above 5% were tested as separate variables in similar stepwise analyses as applied for genotypes. None of the haplotypes qualified into the models.

The two most common haplotypes, H1 and H2, accounted for 56 and 23% of chromosomes, respectively. Interestingly, these two haplotypes only differed at the four sites that we earlier had found to be associated with invasively determined vascular release rates of tPA-antigen, but were equal at the other five sites. When we reanalyzed our earlier experimental data[13] with the haplotypes estimated from the present study, individuals homozygous for H1 had a significantly higher vascular tPA release rate as compared to carriers of H2: 15 *vs* 4 $\text{ng} \times \text{min}^{-1} \times \text{l tissue}^{-1}$, $P<0.001$. Thus, haplotypes provided a better discrimination between high and low tPA secretors than tPA −7,351C>T genotypes. We therefore compared plasma levels of tPA antigen and tPA activity in subjects homozygous for H1 *vs* carriers of H2 in the present study by a similar stepwise model as applied above. However, haplotypes did not qualify into the model.

Discussion

In the present study, we observed the expected positive correlations between plasma tPA antigen and age, blood pressure, metabolic variables, and plasma PAI-1, as well as negative correlations between plasma tPA activity and metabolic variables and PAI-1.[25–29] Observed correlations were very consistent with those earlier reported in a large population sample from the MONICA study in northern Sweden.[25,26] In contrast to these established predictors, genetic variation at the PLAT locus was not a strong predictor for plasma tPA. This is in line with some earlier studies that reported that the tPA Alu insertion polymorphism was not associated with plasma tPA.[30–32] However, in the present study tPA antigen was significantly associated with the tPA 90A>G SNP in an additive model, and tPA activity with the tPA Arg129Trp and 90A>G SNP in the additive and dominant model, respectively. Of the three rare variants that are in LD, the nonsynonomous tPA Arg129Trp is an obvious functional candidate. There are also preliminary data to suggest that the tPA −125T>C SNP might influence DNA–protein binding.[33] However, although significant associations were observed between the rarer SNPs and plasma tPA in the present study, these results should be interpreted with some caution since associations were tested in two different models for each of the two dependent variables. In any case, these rare SNPs only explained a minor proportion of the variation in plasma tPA.

If several sites at a locus contribute to a phenotype, haplotypes may be a better way to detect the genetic contribution than genotypes.[34] However, we did not observe any significant association between the three most common haplotypes and plasma tPA. We have earlier reported an association between SNPs at the PLAT locus and vascular tPA release.[13] When tPA release rate was compared in subjects homozygous for the most common haplotype (H1) defined in the present study with subjects carrying H2, a stronger association emerged than that observed earlier for single SNPs. However, there was no significant association between these two common haplotypes and plasma levels of tPA in either study.

Recent studies show that genetic factors are of importance for the plasma level of tPA antigen.[35–37] Estimates are that the heritability is between 30 and 60%.[35–37] There is also one study reporting a within-family correlation for tPA activity.[38] One might thus argue that some of this genetic variance is attributable to other polymorphisms at the PLAT locus than the ones typed here. However, this seems unlikely since the SNPs that were investigated in the present study were identified when we resequenced

regulatory regions and coding regions of the tPA gene.[13] In addition, five of these SNPs were recently confirmed when 6 kb of the 5′-region and coding regions of the human tPA gene were resequenced in a Japanese population.[39] However, Nakazawa *et al*[39] also identified a novel SNP at −4,358, but given current knowledge about regulation of tPA gene transcription it is unlikely that this site is functional. Thus, it is more likely that the heritability of plasma tPA antigen is explained by variants in other genes, for instance, in pathways related to clearance of tPA and plasma levels of PAI-1. In fact, one previous study reported an association between the PAI-1 −675 4G>5G polymorphism and plasma levels of tPA antigen.[40] Therefore, in addition to the tPA polymorphisms, the PAI-1 polymorphism was genotyped in the present study. However, it did not qualify in any of the models, regardless of whether it was included with or without plasma levels of PAI-1. Consequently, the present results show that the genes responsible for the genetic regulation of plasma tPA remain to be determined.

In the present study only three haplotypes had a frequency above 5% and they represented 88% of the chromosomes. A high LD was also illustrated by the LD coefficients D', which for the majority of pairwise combinations were above 0.7. Similar results were reported from the Japanese study in which 50 unrelated subjects were typed for the tPA Alu insertion polymorphism and four common tPA SNPs.[39] This is consistent with the finding of haplotype blocks, that is, regions with very low haplotype diversity.[41,42] One implication of this is that with knowledge of the haplotype structure at a specific locus in a given population, the essential SNPs for association studies can be identified.[43] Our results show that typing two SNPs (e.g. −7,351C>T and 27,739G>A) was sufficient to define the haplotypes that accounted for 88% of the chromosomes at the PLAT locus.

In conclusion, the results of the present study show that neither genetic variation at the PLAT locus nor the PAI-1 −675 4G>5G polymorphism are strong predictors of plasma tPA levels, suggesting that variations in other genes contribute to the heritability of this phenotype. The results also show that three haplotypes at the PLAT locus accounted for 88% of the chromosomes and that they could be defined by typing only two SNPs.

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References

1 Wun TC, Capuano A: Spontaneous fibrinolysis in whole human plasma. Identification of tissue activator-related protein as the major plasminogen activator causing spontaneous activity *in vitro*. *J Biol Chem* 1985; **260**: 5061–5066.

2 Emeis JJ: Regulation of the acute release of tissue-type plasminogen activator from the endothelium by coagulation activation products. *Ann NY Acad Sci* 1992; **667**: 249–258.

3 Carmeliet P, Collen D: Gene targeting and gene transfer studies of the biological role of the plasminogen/plasmin system. *Thromb Haemost* 1995; **74**: 429–436.

4 Matsuno H, Kozawa O, Niwa M, *et al*: Differential role of components of the fibrinolytic system in the formation and removal of thrombus induced by endothelial injury. *Thromb Haemost* 1999; **81**: 601–604.

5 Meade TW, Ruddock V, Stirling Y, Chakrabarti R, Miller GJ: Fibrinolytic activity, clotting factors, and long-term incidence of ischaemic heart disease in the Northwick Park Heart Study. *Lancet* 1993; **342**: 1076–1079.

6 Ridker PM, Vaughan DE, Stampfer MJ, Manson JE, Hennekens CH: Endogenous tissue-type plasminogen activator and risk of myocardial infarction. *Lancet* 1993; **341**: 1165–1168.

7 Ridker PM, Hennekens CH, Stampfer MJ, Manson JE, Vaughan DE: Prospective study of endogenous tissue plasminogen activator and risk of stroke. *Lancet* 1994; **343**: 940–943.

8 Kooistra T, Schrauwen Y, Arts J, Emeis JJ: Regulation of endothelial cell t-PA synthesis and release. *Int J Hematol* 1994; **59**: 233–255.

9 Degen SJ, Rajput B, Reich E: The human tissue plasminogen activator gene. *J Biol Chem* 1986; **261**: 6972–6985.

10 Henderson BR, Sleigh MJ: TATA box-independent transcription of the human tissue plasminogen activator gene initiates within a sequence conserved in related genes. *FEBS Lett* 1992; **309**: 130–134.

11 Costa M, Shen Y, Maurer F, Medcalf RL: Transcriptional regulation of the tissue-type plasminogen-activator gene in human endothelial cells: identification of nuclear factors that recognise functional elements in the tissue-type plasminogen-activator gene promoter. *Eur J Biochem* 1998; **258**: 123–131.

12 Bulens F, Merchiers P, Ibanez-Tallon I *et al*: Identification of a multihormone responsive enhancer far upstream from the human tissue-type plasminogen activator gene. *J Biol Chem* 1997; **272**: 663–671.

13 Ladenvall P, Wall U, Jern S, Jern C: Identification of eight novel single-nucleotide polymorphisms at human tissue-type plasminogen activator (t-PA) locus: association with vascular tPA release *in vivo*. *Thromb Haemost* 2000; **84**: 150–155.

14 Chandler WL, Alessi MC, Aillaud MF, Henderson P, Vague P, Juhan-Vague I: Clearance of tissue plasminogen activator (TPA) and TPA/plasminogen activator inhibitor type 1 (PAI-1) complex: relationship to elevated TPA antigen in patients with high PAI-1 activity levels. *Circulation* 1997; **96**: 761–768.

15 Wilhelmsen L, Johansson S, Rosengren A, Wallin I, Dotevall A, Lappas G: Risk factors for cardiovascular disease during the period 1985–1995 in Goteborg, Sweden. The GOT-MONICA Project. *J Intern Med* 1997; **242**: 199–211.

16 Minnesota code for resting electrocardiograms. in: Rose GA BH, Gillum RF, Prineas RJ (eds): *Cardiovascular survey methods*. Geneva: World Health Organization, 1982.

17 Ranby M, Nguyen G, Scarabin PY, Samama M: Immunoreactivity of tissue plasminogen activator and of its inhibitor complexes. Biochemical and multicenter validation of a two site immunosorbent assay. *Thromb Haemost* 1989; **61**: 409–414.

18 Meijer P, Pollet DE, Wauters J, Kluft C: Specificity of antigen assays of plasminogen activator inhibitor in plasma: Innotest PAI-1 immunoassay evaluated. *Clin Chem* 1994; **40**: 110–115.

19 de Maat MP, de Bart AC, Hennis BC et al: Interindividual and intraindividual variability in plasma fibrinogen, TPA antigen, PAI activity, and CRP in healthy, young volunteers and patients

with angina pectoris. *Arterioscler Thromb Vasc Biol* 1996; **16**: 1156–1162.
20 Livak KJ, Marmaro J, Todd JA: Towards fully automated genome-wide polymorphism screening. *Nat Genet* 1995; **9**: 341–342.
21 Jern C, Ladenvall P, Wall U, Jern S: Gene polymorphism of t-PA is associated with forearm vascular release rate of t-PA. *Arterioscler Thromb Vasc Biol* 1999; **19**: 454–459.
22 Tjärnlund A, Andersson M, Jood K, Ladenvall P, Jern C: Rapid genotyping of hemostatic gene polymorphisms using the 5′ nuclease assay. *Thromb Haemost* 2003; **89**: 962–968.
23 Lewontin R: The interaction of selection and linkage. I. General considerations; heterotic models. *Genetics* 1964; **49**: 49–67.
24 Terwilliger J, Ott J: Linkage disequilibrium between alleles at marker loci; in: Terwilliger J, Ott J (eds): *Handbook of human genetic linkage*. Baltimore: John Hopkins University Press, 1994: pp 188–198.
25 Eliasson M, Evrin P, Lundblad D, Asplund K, Ranby M: Influence of gender, age and sampling time on plasma fibrinolytic variables and fibrinogen. *Fibrinolysis* 1993; **7**: 316–323.
26 Eliasson M, Evrin PE, Lundblad D: Fibrinogen and fibrinolytic variables in relation to anthropometry, lipids and blood pressure. The Northern Sweden MONICA Study. *J Clin Epidemiol* 1994; **47**: 513–524.
27 Ridker PM, Vaughan DE, Stampfer MJ, Sacks FM, Hennekens CH: A cross-sectional study of endogenous tissue plasminogen activator, total cholesterol, HDL cholesterol, and apolipoproteins A-I, A-II, and B-100. *Arterioscler Thromb* 1993; **13**: 1587–1592.
28 Juhan-Vague I, Thompson SG, Jespersen J: Involvement of the hemostatic system in the insulin resistance syndrome. A study of 1500 patients with angina pectoris. The ECAT Angina Pectoris Study Group. *Arterioscler Thromb* 1993; **13**: 1865–1873.
29 Wall U, Jern C, Bergbrant A, Jern S: Enhanced levels of tissue-type plasminogen activator in borderline hypertension. *Hypertension* 1995; **26**: 796–800.
30 Hooper WC, Lally C, Austin H *et al*: The role of the t-PA I/D and PAI-1 4G/5G polymorphisms in African-American adults with a diagnosis of myocardial infarction or venous thromboembolism. *Thromb Res* 2000; **99**: 223–230.
31 Iacoviello L, di Casteluovo A, de Knijff P, d'Orazio C, Kluft C, Donati M: Alu-repeat polymorphism in the tissue-type plasminogen activator (t-PA) gene, t-PA levels and risk of familial myocardial infarction (MI). *Fibrinolysis* 1996; **10**: 13–16.
32 van der Bom JG, de Knijff P, Haverkate F *et al*: Tissue plasminogen activator and risk of myocardial infarction. The Rotterdam Study. *Circulation* 1997; **95**: 2623–2627.
33 Blaxill J, Futers T, Grant P: A novel tissue plasminogen activator promoter polymorphism: functional characteristics and relationship to atheroma and myocardial infarction. *Thromb Haemost* 2001; **86**: abstract.
34 Drysdale CM, McGraw DW, Stack CB *et al*: Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict *in vivo* responsiveness. *Proc Natl Acad Sci USA* 2000; **97**: 10483–10488.
35 Souto JC, Almasy L, Borrell M *et al*: Genetic determinants of hemostasis phenotypes in Spanish families. *Circulation* 2000; **101**: 1546–1551.
36 de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ: The genetics of haemostasis: a twin study. *Lancet* 2001; **357**: 101–105.
37 Freeman MS, Mansfield MW, Barrett JH, Grant PJ: Genetic contribution to circulating levels of hemostatic factors in healthy families with effects of known genetic polymorphisms on heritability. *Arterioscler Thromb Vasc Biol* 2002; **22**: 506–510.
38 Glueck CJ, Glueck HI, Tracy T, Speirs J, Stroop D: Inheritance conjointly contributing to fibrinolysis and hyperlipidemia. *Metabolism* 1993; **42**: 1410–1419.
39 Nakazawa I, Nakajima T, Ishigami T, Umemura S, Emi M: Linkage disequilibrium and haplotype analysis among eight novel single-nucleotide polymorphisms in the human tissue-type plasminogen activator (t-PA) gene. *J Hum Genet* 2001; **46**: 367–371.
40 Henry M, Tregouet DA, Alessi MC *et al*: Metabolic determinants are much more important than genetic polymorphisms in determining the PAI-1 activity and antigen plasma concentrations: a family study with part of the Stanislas Cohort. *Arterioscler Thromb Vasc Biol* 1998; **18**: 84–91.
41 Daly MJ, Rioux JD, Schaffner SF, Hudson TJ, Lander ES: High-resolution haplotype structure in the human genome. *Nat Genet* 2001; **29**: 229–232.
42 Tiret L, Poirier O, Nicaud V *et al*: Heterogeneity of linkage disequilibrium in human genes has implications for association studies of common diseases. *Hum Mol Genet* 2002; **11**: 419–429.
43 Johnson GC, Esposito L, Barratt BJ *et al*: Haplotype tagging for the identification of common disease genes. *Nat Genet* 2001; **29**: 233–237.