Table 5. Multiple logistic regression analysis

Variables[a]	Category	OR (95% CI)
Model 1		
APOE	*ε4* (−) (Ref)	1.00
	ε4 (+)	**5.00 (4.20–5.96)***
Gender	Male (Ref)	1.00
	Female	**1.64 (1.38–1.94)***
SNP rs713250[b]	TT (Ref)	1.00
	TC	1.13 (0.92–1.37)
	CC	**1.36 (1.08–1.71)****
Age	—	**1.01 (1.00–1.02)*****
Model 2		
APOE	*ε4* (−) (Ref)	1.00
	ε4 (+)	**5.74 (3.62–9.10)***
Gender	Male (Ref)	1.00
	Female	0.88 (0.62–1.26)
SNP rs713250[b]	TT (Ref)	1.00
	TC	0.81 (0.58–1.12)
	CC	0.75 (0.51–1.10)
Age	—	**1.02 (1.01–1.03)****
SNP rs713250_gender[b]	Others (Ref)	1.00
	TC_Female	**1.68 (1.12–2.54)*****
	CC_Female	**2.57 (1.59–4.17)***
Age_*APOE*	Age_*ε4* (−) (Ref)	1.00
	Age_*ε4* (+)	**0.97 (0.95–1.00)*****
Gender_*APOE*	Others (Ref)	1.00
	Female_*ε4* (+)	**1.49 (1.03–2.15)*****

Ref, reference.
*P-value <0.001; **P-value <0.01; ***P-value <0.05.
[a]'_' signifies the interaction between variables.
[b]Global P-value <0.05.

To determine whether or not the difference in the Aβ40/42 ratio between LOAD and the controls is due to the SNPs identified here, two-way ANOVA was performed across diagnosis (LOAD and control) and three genotypic groups (major homozygotes, heterozygotes and minor homozygotes) within different gender and their combined groups (Fig. 3D–F). SNP rs713250 was used as a representative of the seven associated SNPs because it showed the most significant association with LOAD on Mantel–Haenszel test (allelic $P\text{-value}_{MH\text{-}F} = 0.000005945$), as shown in Table 2. The log-transformed Aβ40/42 ratio values [$\log_2$(Aβ40/42 ratio + 1)] were used in this analysis. Before two-way ANOVA, the Kolmogorov–Smirnov (KS) normality test and Bartlett's test for equal variances were performed for the each dataset as to gender. Almost every sub-group examined passed the KS normality test. Both the female–male (Fig. 3D) and female (Fig. 3E) groups passed the Bartlett's test, but not the male group (Fig. 3F, $P = 0.01178$). Through two-way ANOVA, a significant effect of diagnosis was observed for every group (P-values <0.0001). However, we did not detect any genotype-dependent effect of this SNP on the Aβ40/42 ratio, and no interaction between the SNP, Aβ40/42 ratio and diagnosis.

DISCUSSION

In this study, we extended our previous work on chromosome 10q (26), and thoroughly reanalyzed the genotype data for 1140 SNPs in order to discover gender-related genetic loci for LOAD. In a single SNP-based case–control study, we found seven SNPs on *CTNNA3* showing genetic association with LOAD in females with the *APOE-ε3*3* genotype or without the *APOE-ε4* allele. Furthermore, multiple logistic regression analysis revealed that one (SNP rs713250) of these seven SNPs directly interacted with the female gender, but not with the male gender, and did not show any interaction with the *APOE-ε4* allele at all. These are the first findings constituting evidence that *CTNNA3* may affect the development of sporadic LOAD through a novel female-specific mechanism independent of the *APOE-ε4* allele. We consider the genetic association identified here to reflect one single signal. The reasons are: (1) the seven significant SNPs span only ~38 kb and are clustered in intron 9 of *CTNNA3* (Fig. 2A and C), which suggests a multiple-hit genomic region of SNPs associated with LOAD; (2) solid linkage disequilibrium was observed between all of these seven SNPs ($D' > 0.9$) (Supplementary Material, Fig. S1); and (3) the associated region was encompassed by a tight structured LD block extending ~80 kb (Fig. 2B).

Janssens *et al.* (29,30) cloned full-length *CTNNA3* cDNA as a novel member of the α-catenin gene family and determined its genomic structure. *CTNNA3* contains 18 exons and spans ~1.78 Mb (67.35–69.13 Mb), being the longest of all genes located on chromosome 10. The chromosomal location of *CTNNA3* is 10q21 (30), which includes the suggestive linkage region between microsatellite markers D10S1227 (57.20 Mb) and D10S1211 (66.39 Mb) in LOAD (24). Ertekin-Taner *et al.* (23) found a linkage with a maximum LOD score of 3.93 at 81 cM close to D10S1225 (64.43 Mb) using the plasma Aβ42 level as a surrogate trait in a set of LOAD families, and the same chromosomal region was identified by Myers *et al.* (24) by means of genome-wide screening of sibling pairs with LOAD. To date, there have been six papers on the genetic association of *CTNNA3* with LOAD (32–37). In the first report (32), it was demonstrated that two SNPs located in intron 13 of *CTNNA3* are associated with familial LOAD with high levels of plasma Aβ42, which was used as an intermediate phenotype related to AD. These intronic SNPs, spanning 423 bp, are rs12357560 and rs7070570: the former lies 1174 bp upstream, and the latter 1597 bp downstream from exon 14, respectively. They are in strong LD: $D'=1$ in all four populations, CEU, CHB, JPT and YRI, used in the HapMap project (38). A genotype-dependent correlation between SNP rs7070570 and the plasma Aβ42 level has also been detected: the major homozygote (TT) is associated with the highest level of Aβ42, the heterozygote (TC) with an intermediate level and the minor homozygote (CC) with the lowest level (32). Martin *et al.* (34). found that SNP rs7074454 located in intron 13 of *CTNNA3*, lying 355 bp upstream from SNP rs7070570, was significantly associated with both familial and sporadic cases of LOAD. Non-synonymous SNP rs4548513 (AGC → AAC, Ser596Asn) located in exon 13 of *CTNNA3*, lying 175 721 bp upstream from SNP rs7070570, has been shown to be associated with familial AD (37). All of these four SNPs, rs7070570, rs12357560, rs7074454 and rs4548513, lie in a genomic region extending from exons 13 to 14 (Fig. 2A), which has been shown to be located within a large LD block spanning around 310 kb (67.43–67.74 Mb)

Table 6. Case–control haplotype analysis

Sample set	Gender	Number of subjects		Haplotype[a]	Frequency		Number of estimated alleles		Permutation *P*-value (10 000)	OR (95% CI)
		LOAD	Control		LOAD	Control	LOAD	Control		
All	Female	1103	998	**[H1]C-A-T-T-T-A-T**	0.4717	0.5174	1041	1033	**0.0029**	0.83 (0.74–0.94)
				[H2]A-G-C-C-G-G-C	0.3592	0.3375	792	674	0.1538	1.10 (0.97–1.25)
				[H3]A-G-C-T-T-G-C	0.1406	0.1110	310	222	**0.0043**	1.31 (1.09–1.57)
				[H4]C-A-T-T-T-A-C	0.0196	0.0169	43	34	0.5632	1.15 (0.73–1.81)
				Others[b]	0.0089	0.0172	20	33	—	—
				Sum	1.0000	1.0000	2206	1996	—	—
				Global	—	—	—	—	**0.0006**	—
	Male	423	668	[H1]C-A-T-T-T-A-T	0.5293	0.4973	448	664	0.145	1.14 (0.96–1.35)
				[H2]A-G-C-C-G-G-C	0.3344	0.3415	283	456	0.7739	0.97 (0.81–1.16)
				[H3]A-G-C-T-T-G-C	0.1179	0.1314	100	176	0.3927	0.88 (0.68–1.15)
				[H4]C-A-T-T-T-A-C	0.0084	0.0131	7	18	0.3117	0.61 (0.25–1.47)
				Others[b]	0.01	0.0167	8	22	—	—
				Sum	1.0000	1.0000	846	1336	—	—
				Global	—	—	—	—	0.2273	—
Negative-*ε4*	Female	522	827	**[H1]C-A-T-T-T-A-T**	0.4430	0.5228	462	865	**< 0.0001**	0.72 (0.62–0.85)
				[H2]A-G-C-C-G-G-C	0.3888	0.3273	406	541	**0.0008**	1.31 (1.11–1.54)
				[H3]A-G-C-T-T-G-C	0.1418	0.1132	148	187	**0.0323**	1.30 (1.02–1.63)
				[H4]C-A-T-T-T-A-C	0.0206	0.0185	22	31	0.6661	1.13 (0.65–1.96)
				Others[b]	0.0058	0.0182	6	30	—	—
				Sum	1.0000	1.0000	1044	1654	—	—
				Global	—	—	—	—	**0.0008**	—
	Male	227	551	[H1]C-A-T-T-T-A-T	0.5240	0.5039	238	556	0.5078	1.08 (0.87–1.35)
				[H2]A-G-C-C-G-G-C	0.3479	0.3456	158	381	0.9532	1.01 (0.80–1.27)
				[H3]A-G-C-T-T-G-C	0.1167	0.1289	53	142	0.5618	0.89 (0.64–1.25)
				Others[b]	0.0114	0.0216	5	23	—	—
				Sum	1.0000	1.0000	454	1102	—	—
				Global	—	—	—	—	0.7917	—
*ε3*3*	Female	491	748	**[H1]C-A-T-T-T-A-T**	0.4363	0.5179	428	775	**0.0002**	0.72 (0.61–0.85)
				[H2]A-G-C-C-G-G-C	0.3919	0.3305	385	494	**0.0019**	1.31 (1.11–1.55)
				[H3]A-G-C-T-T-G-C	0.1436	0.1151	141	172	**0.0405**	1.29 (1.02–1.64)
				[H4]C-A-T-T-T-A-C	0.0219	0.0178	22	27	0.4617	1.25 (0.71–2.20)
				Others[b]	0.0063	0.0187	6	28	—	—
				Sum	1.0000	1.0000	982	1496	—	—
				Global	—	—	—	—	**0.001**	—
	Male	208	495	[H1]C-A-T-T-T-A-T	0.5214	0.4995	217	491	0.383	1.11 (0.88–1.39)
				[H2]A-G-C-C-G-G-C	0.3459	0.3525	144	349	0.8585	0.97 (0.76–1.24)
				[H3]A-G-C-T-T-G-C	0.1202	0.1300	50	129	0.6659	0.91 (0.64–1.29)
				Others[b]	0.0125	0.0220	5	21	—	—
				Sum	1.0000	1.0000	416	990	—	—
				Global	—	—	—	—	0.8879	—
Positive-*ε4*	Female	581	171	[H1]C-A-T-T-T-A-T	0.4976	0.4907	577	168	0.9006	1.02 (0.80–1.30)
				[H2]A-G-C-C-G-G-C	0.3327	0.3870	387	132	0.0799	0.79 (0.62–1.02)
				[H3]A-G-C-T-T-G-C	0.1396	0.1009	162	35	0.0797	1.42 (0.96–2.09)
				[H4]C-A-T-T-T-A-C	0.0187	0.09	22	3	0.2313	2.18 (0.65–7.33)
				Others[b]	0.0114	0.0124	14	4	—	—
				Sum	1.0000	1.0000	1162	342	—	—
				Global	—	—	—	—	0.3323	—
	Male	196	117	[H1]C-A-T-T-T-A-T	0.5356	0.4638	210	109	0.0961	1.32 (0.96–1.83)
				[H2]A-G-C-C-G-G-C	0.3188	0.3238	125	76	0.934	0.97 (0.69–1.38)
				[H3]A-G-C-T-T-G-C	0.1193	0.1459	47	34	0.3988	0.80 (0.50–1.29)
				[H4]C-A-T-T-T-A-C	0.0129	0.0310	5	7	0.1429	0.42 (0.13–1.34)
				Others[b]	0.0134	0.0355	5	8	—	—
				Sum	1.0000	1.0000	392	234	—	—
				Global	—	—	—	—	0.0728	—

Statistically significant haplotypes and permutation *P*-values are highlighted in bold.
[a]The SNP order, from left to right, is as follows: rs7909676, rs2394287, rs4459178, rs10997307, rs12258078, rs10822890 and rs713250.
[b]Haplotypes with frequencies <0.01 in both LOAD and control subjects.

in CEU subjects (37) (Supplementary Material, Fig. S2). They have a tendency to exhibit selective association with familial rather than sporadic LOAD (32,35,37). Therefore, it is likely that the large LD block region contributes to a specific form of familial LOAD in Caucasians. We also assessed these four SNPs and SNPs neighboring them in our Japanese sporadic LOAD subjects, however, none of these SNPs exhibited significant association (data not shown). In the genomic

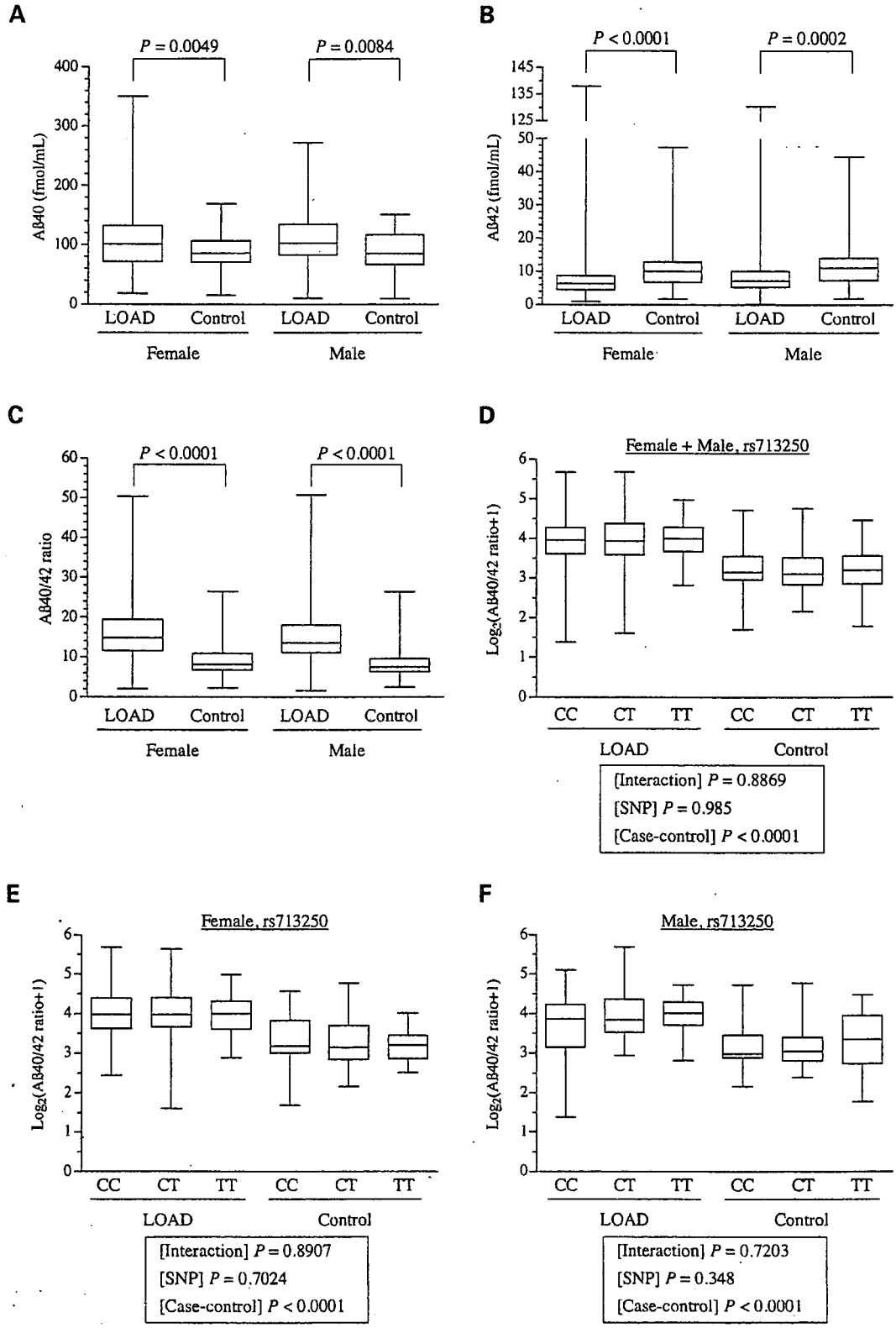

Figure 3. Comparison of the plasma levels of Aβ40 and Aβ42, and the Aβ40/42 ratio. The differences in the relative amounts of Aβ40 (**A**) and Aβ42 (**B**), and the Aβ40/42 ratio (**C**) were compared between LOAD patients and controls by means of Mann–Whitney's *U*-test within different gender groups. (**D**, **E**, **F**) Correlation between the Aβ40/42 ratio, an associated SNP on *CTNNA3*, and the diagnosis (LOAD or control). Using log-transformed Aβ40/42 ratio values, two-way ANOVA tests were performed after Bartlett's test for the homogeneity of variances and the KS normality test. The results for SNP rs713250 are presented here as being representative of the seven associated SNPs identified in this study. The horizontal line inside each box denotes the median value. The box extends from the 25th and 75th percentiles. The error bars extend down to the lowest value and up to the highest. Genotypes CC, CT and TT represent major-allele homozygotes, heterozygotes and minor-allele homozygotes, respectively.

region including the four SNPs, different LD block structures were observed in Japanese and CEPH subjects (Fig. 2B and Supplementary Material, Fig. S2). As one of the reasons why reproducible association could not be detected for these four SNPs, we mainly consider that an ethnic difference may exist.

High-level gene expression of *CTNNA3* is detected predominantly in heart and testis, and low-level expression in several tissues including brain (29). Coimmunoprecipitation analysis revealed that CTNNA3 binds directly to β-catenin in both a human cell line transfected with *CTNNA3* cDNA, and heart and testis tissue extracts of mouse (30). β-Catenin forms a complex with presenilin 1 (PSEN1) (31,39,40), mutations of which cause familial cases of early-onset AD (EOAD) [Alzheimer Disease & Frontotemporal Dementia Mutation Database (AD&FTDMDB), http://www.molgen.ua.ac.be/ADMutations/]. The expression level of β-catenin is reduced in the brains of EOAD patients with *PSEN1* mutations (31). Intracellular trafficking of β-catenin is affected in human cells bearing *PSEN1* mutations (41), resulting in sustained loss of Wnt/β-catenin signal transduction, which is probably followed by the onset and development of AD (42,43). Although, at present, there is no direct evidence suggesting that CTNNA3 interacts with PSEN1, it is assumed that their genetic polymorphisms or combinations in *CTNNA3* may have a negative influence on the Wnt/β-catenin signaling pathway, leading to potential involvement in the pathogenesis of AD. In this study, it was clarified that seven intronic SNPs on *CTNNA3* were significantly and reproducibly associated with sporadic female cases of LOAD without the *APOE-ε4* allele. Intronic variants are considered to have the potential to directly affect gene-expression levels in some cases (44); therefore, we performed quantitative real-time RT–PCR analysis of *CTNNA3* using the postmortem brains of 19 neuropathologically-confirmed LOAD cases and 22 control ones. Two-way ANOVA revealed that there was no statistically significant interaction between the *CTNNA3* expression level, the associated SNPs identified here and the diagnosis (data not shown). Additionally, although a genotype-dependent transition effect on the plasma Aβ42 level was observed for intronic SNP rs7070570 by Ertekin-Taner *et al.* (32), it was found that none of these SNPs influence the plasma levels of Aβ peptides (Fig. 3D–F).

However, interestingly, by means of a search of a public genome database, the Database of Genomic Variants (http://projects.tcag.ca/variation/), we discovered that there is copy number variation (CNV) (45) in the genomic region comprising the seven associated SNPs on *CTNNA3*: variation ID 3807 at Locus 2128, which was detected in a Japanese subject (ID, NA18973) (Fig. 2A). CNV, i.e. deletion, insertion and duplication with >1 kb in length of the genomic sequence (46), rather than SNP could cause phenotypic diversity and complex diseases in humans by altering the gene dose or disrupting the coding or regulatory sequences of genes, and may account for the LOAD susceptibility. Regarding our LOAD subjects, we did not examine the presence or absence of CNV within *CTNNA3*. Therefore, in a further study, it is very important to determine whether or not CNV in *CTNNA3* is associated with LOAD.

Recently, in LOAD families, notable evidence was obtained suggesting a maternal parent-of-origin effect on chromosome 10q between microsatellite markers D10S1233 (44.05 Mb) and D10S1225 (64.43 Mb) with a non-parametric LOD score >1.0: the highest LOD score of 3.73 was seen for microsatellite marker D10S1221 (57.20 Mb) (27,28). Moreover, it was found that *CTNNA3* is subject to genomic imprinting with cell-type specificity in placental tissues: biallelic and monoallelic (maternal-allele) expression is observed in extravillus and villus trophoblasts, respectively (47). Mouse *Ctnna3* (Clone ID 4933408A16 on FANTOM2), orthologous to human *CTNNA3*, has been deposited as a maternal imprinting gene on chromosome 10 in the Expression-based Imprint Candidate Organizer DataBase (48; EICO DB, http://fantom2.gsc.riken.jp/EICODB/imprinting/), provided by RIKEN (Japan). These findings led us to examine whether or not *CTNNA3* shows allele-specific expression caused by a molecular mechanism such as genomic imprinting in the brain. We conducted real-time RT–PCR analysis with allele-specific amplification using postmortem human brains heterozygous for non-synonymous SNP rs4548513 in exon 13 [LOAD, 7 (female:male = 3:4); control, 8 (female:male = 3:5)]. Unexpectedly, biallelic expression was detected in brain tissues, and there was no significant difference between LOAD patients and control subjects in the expression level of *CTNNA3* (data not shown). Since as in placental tissues, as described above, it is possible that cell-type dependent imprinting for *CTNNA3* may occur in the brain, further expression analysis should be carefully carried out using homogeneous populations of specific cells from brain tissues. Now genome-wide prediction and the discovery of imprinted genes have progressed (49,50), and 600 (2.5%) of 23 788 annotated autosomal genes have been found to be potentially imprinted in the mouse genome by computational estimation: 384 (64%) of these candidate-imprinted genes show maternal-allele expression (50). It is expected that failure of imprinted gene expression in the human brain may lead to cognition and behavior defects such as Alzheimer's disease, schizophrenia, the bipolar affective disorder and epilepsy (51–53). Therefore, it is important and interesting to actively examine imprinted genes present in the genetic linkage region of LOAD.

MATERIALS AND METHODS

Subjects

The Japanese Genetic Study Consortium for AD (JGSCAD) was organized in 2000, and blood samples were collected to survey risk genes for LOAD by means of a genome-wide association study. All individuals included in this study were Japanese. Probable AD cases met the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders. Control subjects who had no signs of dementia and lived in an unassisted manner in the local community were also recruited. Age at onset (AAO) is here defined as the age at which the family and/or individuals first noted cognitive problems during work or in daily activities. The Mini-Mental State Examination (MMSE), and Clinical Dementia Rating and/or the Function Assessment Staging were used for the evaluation of cognitive impairment: MMSE was used for almost every subject.

The basic demographics of the LOAD patients and non-demented control subjects are presented in Table 1. A total of 3192 subjects comprising 1526 LOAD patients [female, 1103 (72.3%); male, 423 (27.7%)] and 1666 controls [female, 998 (59.9%); male, 668 (40.1%)], which is referred to as overall sample set All in this study, were used to discover gender-related loci associated with LOAD on chromosome 10q: information on these subjects was also presented in our recent paper, Kuwano *et al.* (26). The mean AAO ± standard deviation (SD) in the 1526 LOAD patients was 73.5 ± 6.6 (range 60–93). The mean age at examination (AAE) ± SD of the control subjects was 73.1 ± 7.8 (range 60–96). There was no significant difference between AAO in LOAD patients and AAE in control subjects with the unpaired Student's *t*-test (*P*-value = 0.1239). The mean MMSE score in the 1526 LOAD patients was 16.5 (SD 7.0), which was significantly lower (*P*-value with unpaired Student's *t*-test <0.0001) than that in the 1666 controls (mean ± SD 28.0 ± 1.8). The numbers (frequency) of *APOE-ε2*2*, *ε2*3*, *ε2*4*, *ε3*3*, *ε3*4* and *ε4*4* in the 1526 LOAD subjects were 1 (0.07%), 49 (3.21%), 17 (1.11%), 699 (45.81%), 613 (40.17%) and 147 (9.63%), and those in the 1666 control subjects were 3 (0.18%), 132 (7.92%), 15 (0.90%), 1243 (74.61%), 256 (15.37%) and 17 (1.02%). The allelic distribution of *APOE* was significantly different between LOAD patients (*ε2*, 68; *ε3*, 2060; *ε4*, 924) and control subjects (*ε2*, 153; *ε3*, 2874; *ε4*, 305), as expected (*P*-value with χ^2 test using a 2×3 contingency table, <0.0001).

The present study was approved by the Institutional Review Board of Niigata University and by all participating institutes. Informed consent was obtained from all controls and appropriate proxies for patients, and all samples were anonymously analyzed for genotyping.

SNPs and genotyping

SNP information was obtained from five open databases: NCBI dbSNP (Build 125, http://www.ncbi.nlm.nih.gov/SNP/), UCSC Genome Bioinformatics (http://genome.ucsc.edu/), International HapMap Project (Rel#20/phaseII on NCBI Build 35.1 assembly and dbSNP Build 125, http://www.hapmap.org/index.html), Ensemble Human (Version 37 on NCBI Build 35.1, http://www.ensembl.org/Homo_sapiens/) and Celera myScience (Version R27 g on NCBI Build 35.1, http://myscience.appliedbiosystems.com/). We selected 1322 SNPs in the region from 60 to 107 Mb on chromosome 10q; mean intermarker distance ± SD, 34.9 ± 87.4 kb; 95% CI, 30.2–39.6 kb. The information on all SNPs, including rs or Celera IDs and genomic positions on NCBI build 35.1, used here was presented in detail elsewhere (26). These SNPs consisted of 29 missense mutations, 27 silent mutations, 6 SNPs in the 5′-UTR, 29 SNPs in the 3′-UTR, 921 SNPs in introns, 282 SNPs in intergenic regions and 28 SNPs in four loci shared by two different genes (*CTNNA3/LRRTM3*, *CDH23/C10orf54*, *C10orf55/PLAU* and *PGAM1/EXOSC1*). Among the 1322 SNPs, 28 SNPs could not be genotyped. To examine deviation from HWE of 1294 SNPs, exact tests (details given under Statistical analysis) were performed with both 363 LOAD patients and 337 control subjects (carrying *APOE-ε3*3* in the exploratory sample set, as shown in Table 1). We used 1140 SNPs that were shown to be actually polymorphic in the Japanese population and showed *P*-values >0.05 with the exact tests; mean intermarker distance ± SD, 40.5 ± 96.7 kb; 95% CI, 34.9–46.1 kb.

Genomic DNA was extracted from peripheral blood with a QIAamp DNA Blood Maxi Kit (Qiagen, Duseldorf, Germany) and examined fluorometrically with a PicoGreen dsDNA quantification kit (Molecular Probes, California, USA). SNP genotyping of individual samples was performed with an ABI PRISM 7900HT instrument using TaqMan technology, and TaqMan SNP Genotyping Assays were purchased from Applied Biosystems (California, USA).

Case–control study

To discover gender-related genetic loci on chromosome 10q (60–107 Mb on NCBI build 35.1), allelic association was assessed by means of the χ^2 test based on a 2×2 contingency table in comparison with allele frequencies in LOAD patients and control subjects within different gender groups. For screening, two independent sample sets, Exploratory and Validation, comprising case–control subjects with *APOE-ε3*3* were first used after being stratified as to gender (Table 1). Sample set Exploratory comprising 363 LOAD patients and 337 control subjects was genotyped (26), and SNPs showing significant association (allelic *P*-value <0.01) were then subjected to further examination using another sample set, Validation, comprising 336 LOAD patients and 372 control subjects. Multistage, including two-stage, genotyping designs for large-scale association surveys have been proved to be practically as well as theoretically effective for identifying common genetic variants that predispose to human disease (54–58). Therefore, we considered that replication in both the Exploratory and Validation sample sets implicates an association of particular SNPs with LOAD.

Subsequently, for stratified analysis we increased the number of subjects and constructed an overall sample set, All. Furthermore, to construct three sub-sample sets, overall sample set All was stratified as to the *APOE* carrier status: Negative-*ε4*, *APOE-ε 2*2*, *2*3* and *3*3*; *ε3*3*, *APOE-ε 3*3*; Positive-*ε4*, *APOE-ε 2*4*, *3*4* and *4*4* (Table 1). The sample numbers for LOAD patients and controls in All, Negative-*ε4*, *ε3*3* and Positive-*ε4* were 1526 and 1666, 749 and 1378, 699 and 1243, and 777 and 288, respectively. These four sample sets were used for the χ^2 test after being sub-grouped as to gender.

Case–control haplotype analysis with significant SNPs was also performed using the following sample sets: All, Negative-*ε4*, *ε3*3* and Positive-*ε4*. These four sample sets were used after being stratified as to gender.

Aβ40 and Aβ42 quantification

For Aβ40 and Aβ42 quantification, 603 subjects consisting of 456 LOAD patients (female, 332; male, 124) and 147 control subjects (female, 95; male, 52) were used. They are included in the All set. The sandwich enzyme-linked immunosorbent assay (59–61) was used to specifically quantify whole plasma Aβ species. The standardization, sensitivity and specificity of the method were described in a previous paper (61).

Briefly, microplates (Immunoplate I; Nunc, Rockilde, Denmark) were pre-coated with monoclonal BNT77 (IgA isotype specific for Aβ11–16) and then sequentially incubated for 24 h at 4°C (100 μl of whole plasma/well), followed by 24 h incubation at 4°C with horseradish-peroxidase-conjugated BA27 (anti-Aβ1–40, specific for Aβ40) or BC05 (anti-Aβ35–43, specific for Aβ42). Color was developed with 3,3′,5,5′-tetramethylbenzidine and evaluated at 450 nm with a microplate reader (Molecular Devices, CA). Synthetic Aβ40 and Aβ42 (Sigma, St Louis, MO) of known concentration (estimated from the amino acid composition) were used as standards. The plates were normalized as to each other by inclusion of three standard plasma samples on all plates.

Statistical analysis

Allele frequencies were calculated by allele counting. To evaluate deviation from the HWE of each SNP marker, we carried out an exact test (62) based on the probability of occurrence of genotypic contingency tables with fixed total numbers of alleles within each sample set (LOAD patients and controls included in two screening sets, Exploratory and Validation). For single SNP case–control analysis, the allelic distributions in LOAD patients and controls were compared by means of χ^2 tests via standard 2×2 contingency tables. Evidence of replication, rather than multiple testing corrections, was used to evaluate the significance of associated SNPs. To comprehensively assess the reproducible SNPs, we conducted a Mantel–Haenszel test, where Exploratory and Validation samples in our case–control study were considered as the strata (63), and computed pooled ORs with 95% CI and *P*-values from Mantel–Haenszel statistics (Statcel 2; OMS, Tokyo, Japan). Estimation of haplotypes and their frequencies was carried out for LOAD patients and controls separately by the maximum-likelihood method from unphased diploid genotype data using an EM algorithm (64) with the following parameters: iteration counter, 5000; conversion criterion, 0.000001. To assess the differences in haplotype distribution between LOAD patients and controls, a permutation test (65) was performed. In this test, all permutation *P*-values were empirically computed using 10 000 iterations of random sampling with fixed total numbers of both LOAD and control subjects. OR (95% CI), as an estimate of the relative risk of disease, of each marker or haplotype was calculated from a 2×2 contingency table. For all statistical methods mentioned above, except the Mantel–Haenszel test, we used SNPAlyze software versions 3.2.3 or 6.0.1 (DYNACOM, Chiba, Japan; http://www.dynacom.co.jp/). For calculation of LD measures (D′) and LD block definition by Gabriel *et al.*'s method (66), we used Haploview version 3.32 (67, http://www.broad.mit.edu/mpg/haploview/index.php).

Using SPSS version 13.0 software (SPSS, Chicago, USA), multiple logistic regression analysis (Table 5) was performed to reveal the effects of the *APOE-ε4* [non-carrier of the *ε4* allele (*ε2*2*, *ε2*3* and *ε3*3*)/carrier of the *ε4* allele (*ε2*4*, *ε3*4* and *ε4*4*)], gender (male/female), age and significant SNPs identified here (major-allele homozygote/heterozygote/minor-allele homozygote) on the risk for LOAD as well as their second-order interaction terms. The strength of association between these variables and disease status (control/LOAD) was evaluated with ORs with 95% CI, based on Wald statistics. We examined the four variables by means of a two-step multiple logistic regression analysis according to Akazawa *et al.* (68). In order to examine which variables explain an association with LOAD independently, we initially carried out stepwise logistic regression analysis (forward selection method) without interaction terms. A significance level of 0.05 was used to enter a variable in the model. Through this analysis, the following multiple logistic regression model was fitted (Model 1 in Table 5): $\log(P/(1-P)) = \alpha + \beta_1 X1 + \beta_2 X2 + \beta_3 X3 + \beta_4 X4$, where P denotes the probability of having LOAD, α is the intercept, β_i represents the estimated parameters and Xj the independent variables ($X1$, *APOE-ε4*; $X2$, gender; $X3$, age; $X4$, SNP). We next analyzed the four variables including their second-order interaction terms (SNP_gender, SNP_*APOE-ε4*, SNP_age, gender_*APOE-ε4*, gender_age and age_*APOE-ε4*) by means of a forward stepwise regression method with a significance level of 0.05 for the inclusion of a variable in the model. As a result, the following model was fitted (Model 2 in Table 5): $\log(P/(1-P)) = \alpha + \beta_1 X1 + \beta_2 X2 + \beta_3 X3 + \beta_4 X4 + \beta_5 X5 + \beta_6 X6 + \beta_7 X7$, where P denotes the probability of having LOAD, α is the intercept, β_i represents the estimated parameters and Xj the independent variables ($X1$, *APOE-ε4*; $X2$, gender; $X3$, age; $X4$, SNP; $X5$, SNP_gender; $X6$, gender_*APOE-ε4*; $X7$, age_*APOE-ε4*). Subjects with undetermined SNP genotype data were omitted for multiple logistic regression analysis.

The Mann–Whitney *U*-test was applied to compare differences in the levels of Aβ40 and Aβ42, and their ratio (Aβ40/42) between LOAD patients and controls (Prism 4.0b; GraphPad Software, CA, USA). After Bartlett's test for the homogeneity of variances (Statcel 2) and the KS normality test (Prism 4.0b), the effects of three SNP genotypes (minor-allele homozygotes, heterozygotes and major-allele homozygotes) in three sub-groups stratified as to gender (female–male mixture, female or male) were examined as to levels of the plasma Aβ40/42 ratio using two-way ANOVA (Prism 4.0b). To create more normally distributed datasets, the Aβ40/42 ratio was subjected to log transformation [$\log_2$(Aβ40/42 ratio + 1)] before the two-way ANOVA.

The statistical significance was set at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Plasma Levels of Unactivated Thrombin Activatable Fibrinolysis Inhibitor (TAFI) Are Down-Regulated in Young Adult Women: Analysis of a Normal Japanese Population

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Abstract: **Thrombin-activatable fibrinolysis inhibitor (TAFI) is an anaphylatoxin-inactivating enzyme generated by proteolytic cleavage of its zymogen, and is the same enzyme as that first designated by our group as procarboxypeptidase R (proCPR). TAFI in plasma is presumed to influence vascular disease in its role as a fibrinolysis inhibitor. The activity of TAFI is strongly influenced by genetic polymorphism, especially at amino acids Thr/Ala-147 and Thr/Ile-325. In this study, we analyzed 202 healthy controls who were not on any medication, had no unusual medical history and whose blood data were normal. In a previous report, we established an enzyme-linked immunosorbent assay (ELISA) specific for non-activated TAFI (proCPR), and investigated levels of unactivated TAFI as an estimate of anti-fibrinolytic capacity. In this study, we determined normal Japanese TAFI levels for each age, sex, and genetic polymorphism of Thr/Ala-147 and Thr/Ile-325, and also showed that the TAFI level in young adult women is lower than in aged women.**

Key words: **Pro-carboxypeptidase R (proCPR), Thrombin-activatable fibrinolysis inhibitor (TAFI), Polymorphism, Enzyme-linked immunosorbent assay (ELISA)**

Inflammation and coagulation/fibrinolysis are parallel processes that occur in microvessels and tissues. Plasma carboxypeptidases that play a key role in these events are carboxypeptidase N (CPN) and R (CPR). CPN was reported as a kininase that rapidly inactivates bradykinin and related peptides by cleaving arginine from the COOH-terminal end of peptides in plasma (15). This enzyme has been proposed to regulate activity of various physiologically active peptides such as the anaphylatoxins C3a and C5a (7, 11), fibrinopeptides (37) and plasmin-degradation products of fibrin (4).

CPR, the carboxypeptidase which our group found in fresh serum (9), cleaves arginine and lysine from the COOH-terminal end of peptides and is generated from its zymogen (proCPR) by proteolytic enzymes such as trypsin, thrombin and plasmin (10, 14, 34). CPR has also been reported independently by others who termed it carboxypeptidase U (CPU) (18, 19) and plasma carboxypeptidase B (CPB) (14). Six years later, Bajzar et al. described this protein as thrombin-activatable fibrinolysis inhibitor (TAFI), since when activated, it inhibits the lysis of clots formed during thrombin activation by removing carboxyterminal lysine residues from plasminogen-binding sites (2).

Plasma carboxypeptidase regulates anaphylaxis and fibrinolysis. In a previous study, total TAFI (TAFI+TAFIa) levels were determined using an

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Abbreviations: Af, atrial fibrillation; CLP, caecal ligation and puncture; CPB, carboxypeptidase B; CPN, carboxypeptidase N; CPU, carboxypeptidase U; DIC, disseminated intravascular coagulation; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; HL, hyperlipidemia; HRP, horseradish peroxidase; HT, hypertension; HWE, Hardy-Weinberg equilibrium; mAb, monoclonal antibody; PCR-RFLPs, polymerase chain reaction-restriction fragment length polymorphisms; proCPR, procarboxypeptidase R; SNPs, single nucleotide polymorphisms; TAFI, thrombin-activatable fibrinolysis inhibitor; T/TM, thrombin-thrombomodulin complex; UTR, untranslated region.

enzyme-linked immunosorbent assay (ELISA) and activity levels were assessed using an enzymatic assay. Disturbances in TAFI levels and activity may represent a risk factor in vascular disorders, and several reports have appeared on the relationship between TAFI and deep vein thrombosis (23, 39), disseminated intravascular coagulation (DIC) (40) and coronary artery disease (22, 33, 35, 42). These various analyses provided total TAFI levels and did not distinguish between the activated (TAFIa) and unactivated (TAFI) forms. In order to dissect events with respect to TAFI activation in the acute phase, we established a TAFI sandwich ELISA system that is proCPR-specific (TAFI without TAFIa) (15, 36).

From a genetic standpoint, the levels of circulating TAFI are strongly influenced by polymorphisms in the promoter and the 3' untranslated region (UTR) of the TAFI gene (20) and may have an effect on the risk of venous thrombophilia (16). Several investigators have reported functional polymorphism in the promoter region as well as in the exon at amino acid positions 147 (41) and 325 (32).

The ELISA system currently in use determines total TAFI with a polyclonal antibody. Reports presented in a recent congress have raised questions regarding antibody reactivity to different TAFI isoforms, and it has been suggested that genotype-dependent variation in the TAFI concentration may result in assay artefacts. It has been suggested that genotypic variability may affect enzymatic activity as well as the results obtained with the existing ELISA system (17). In this study, we analyzed the plasma proCPR levels with a sandwich ELISA using monoclonal antibodies and correlated results with Thr147Ala and Thr325Ile polymorphisms in samples from 202 healthy people chosen from 681 donors.

Materials and Methods

Patients. The subjects of this study were chosen from 681 donors and consisted of 202 healthy controls who were not on any medication, had no unusual medical history and whose blood data, complete blood cell counts and results of routine biochemical examination were normal. We obtained the agreement of the patients and volunteers for use of their samples in biochemical, molecular biological and genomic research. Written informed consent was obtained from each individual according to a protocol approved by the Ethics Committee of the Choju Medical Institute on June 2nd, 2000.

After interviewing patients and their families, we excluded individuals that had been diagnosed with or taken medication for diseases associated with problems of coagulation, fibrinolysis and inflammation, for example, diabetes mellitus (DM), valvular problems, atrial fibrillation (Af), hyperlipidemia (HL), severe hypertension (HT), infection, malignancies, and hepatic and renal disease. Routine biochemical and blood cell analyses were performed by the Tousan Labo Center (Toyohashi, Japan).

Collection of blood materials. Midmorning (around 10 AM) blood samples from patients and volunteers were directly collected into vacuum tubes (TERUMO, Tokyo) containing EDTA 2Na and kept on ice. The blood was centrifuged at 3,000 rpm for 20 min at 4 C, and each plasma supernatant was transferred to several Eppendorf tubes for ELISA. Each blood cell pellet was also transferred to an Eppendorf tube for genomic analysis. These samples were stored at −30 C until assayed.

Genomic analysis of TAFI polymorphisms Thr147Ala and Thr325Ile. For investigation of genomic polymorphism in blood cell pellets, we used Ampdirect for Human Blood from the Analytical Instrument Division of Shimadzu Corporation (Kyoto, Japan). *Taq* DNA polymerase was obtained from TaKaRa (Shiga, Japan). The restriction enzyme, *Bbv*I, was from New England Biolabs (Beverly, Mass., U.S.A.) and *Spe*I was from TaKaRa. For electrophoresis, 1.2% Agarose LO3 was purchased from TaKaRa.

Genomic TAFI gene polymorphisms could easily be detected by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs) using the restriction enzymes *Bbv*I for TAFI-147 and *Spe*I for TAFI-325. Sequences of the TAFI-147 and -325 regions were retrieved from GenBank (accession numbers AL137141 and AL157758). PCR was carried out in a 25 μl reaction volume containing a standard reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μM of each dNTP, 10 μM of each primer, 0.5 U *Taq* DNA polymerase) and 50 ng genomic DNA as a template. The protocol consisted of 40 cycles of 94 C for 30 sec, 62 C for 60 sec, and 72 C for 1 min. The TAFI-147 PCR product size was 456 bp, and the G (Ala) allele was digested with *Bbv*I into 28 + 124 + 304 bp, whereas the A (Thr) allele was digested into 28 + 428 bp. The TAFI-325 PCR product was 363 bp, and the C (Thr) allele was digested with *Spe*I into 118 + 245 bp whereas the T (Ile) allele was not digested at all by *Spe*I. PCR products were digested with each enzyme, resolved by electrophoresis on a 10% acrylamide gel, and visualized by ethidium bromide staining (data not shown).

Measurement of proCPR in plasma. Ninety-six-well microtiter plates were coated with 10 μg/ml of a mono-

clonal antibody (mAb) (2A16) against total CPR, which was obtained from the Institute for Protein Science Co., Ltd. (Nagoya, Japan).

After washing with PBS-Tween, 50 μl of 1/400 diluted plasma were added and the plates were left at room temperature for 1 hr. Following an additional PBS-Tween washing, 50 μl of 100 ng/ml of another horseradish peroxidase (HRP)-conjugated mAb against proCPR (10G1) were added. After 1 hr at room temperature, the plates were washed and the OD at 492 nm was determined. Each plate was also treated with purified proCPR obtained from Haematologic Technologies, Inc. (Essex Jct.,Vt., U.S.A.) as a standard.

Statistical analysis. Statistical analysis was carried out on a personal computer running the Windows XP system. The significance of difference for each genotype was examined with both the χ^2 test with Yates's correction and Fisher's exact test using 2 × 2 tables. The level of significance was taken at $P<0.05$.

Results

Genetic Distribution in All Subjects and in Normal Subjects Only

We first analyzed a total of 681 samples. As shown Table 1a, the 229 males (aged 62.0 ± 19.93) and 452 females (66.7 ± 21.94) included both patients and normal volunteers. The 229 males consisted of 88 hospitalized patients and 141 volunteers. The 452 females consisted of 190 hospitalized patients and 262 volunteers. The genotype distribution of each group, excluding Thr/Ile-325 females, did not deviate significantly from Hardy-Weinberg equilibrium (HWE). The volunteers included patients' guardians, hospital staff and people desiring a health examination. However, some volunteers were on medication, showed abnormal biochemical data or had various disqualifying conditions. Finally, 202 healthy volunteers were selected for the analysis.

The 202 normal individuals included 73 males (aged 45.4 ± 18.27, ranging from 20–93 years) and 129 females (aged 43.9 ± 17.53, ranging from 18–90 years). The Thr/Ala-147 female and Thr/Ile-325 male groups deviated slightly from HWE, but overall, the genotype distribution revealed no significant differences. In Table 1b, significant deviation between male and female groups is seen only with the Thr147Ala genotype (among the three groups ($P=0.0096$) and among its alleles ($P=0.0396$)). This tendency was also evident on analysis of the total 681 samples (Table 1a). However, when the data were separated into in-patients and volunteers, no statistical significance was noted at Thr147Ala between the male and female in-patient groups (three groups; $P=0.579$).

Plasma TAFI Level in Each Age Group

The male volunteers were 20–93 years of age and the female volunteers were 18–90, with ages ranging mainly from 20–70 years. The plasma TAFI (proCPR) level of the volunteers was in the range of 10–25 mg/liter. Among the age groups shown in Table 2a, differences between males and females aged 21–30 and 41–50 years were statistically significant (21–30 years; $P=0.00045$, 41–50s; $P=0.010$). The difference in the total average TAFI level for each sex was also statistically significant ($P=0.0001$), which was due primarily to the females under 50 (Fig. 1b). The greatest difference was observed between those younger and older

Table 1a. Sex distribution of the total 681 subjects and Thr/Ala-147 and Thr/Ile-325 polymorphisms

	Males	Females	Total
Number	229	452	681
Age (years)	62.0 ± 19.93	66.7 ± 21.94	65.1 ± 21.38
Thr147Ala		*	
Thr/Thr	17	34	51
Thr/Ala	120	180	300
Ala/Ala	92	238	330
		**	
Allele Thr	154 (33.6%)	248 (27.4%)	402 (29.5%)
Allele Ala	304 (66.4%)	656 (72.6%)	960 (70.5%)
Thr325Ile			
Thr/Thr	157	316	473
Thr/Ile	61	109	170
Ile/Ile	11	27	38
Allele Thr	375 (81.9%)	741 (82.0%)	1,116 (81.9%)
Allele Ile	83 (19.1%)	163 (18.0%)	246 (19.1%)

Difference at Thr147Ala between males and females, $*P < 0.01$, $**P < 0.05$.

Table 1b. Sex distribution and plasma TAFI levels of the normal 202 subjects and Thr/Ala-147 and Thr/Ile-325 polymorphisms

	Males		Females		Total	
	N	mean ± SD (mg/liter)	*N*	mean ± SD (mg/liter)	*N*	mean ± SD (mg/liter)
Thr147Ala						
Thr/Thr	6 (10%)	21.7 ± 4.67	11 (9%)	19.5 ± 3.42	17 (8%)	20.3 ± 3.91
Thr/Ala	44 (70%)	18.1 ± 3.55	50 (39%)	17.7 ± 4.30	94 (47%)	17.9 ± 3.95
Ala/Ala	23 (20%)	16.2 ± 3.60	68 (52%)	15.2 ± 3.41	91 (45%)	15.5 ± 3.45
Allele Thr	56 (38.4%)		72 (45.6%)		128 (31.7%)	
Allele Ala	90 (61.6%)		186 (54.4%)		276 (68.3%)	
Thr325Ile						
Thr/Thr	53 (73%)	18.9 ± 3.84	96 (74%)	17.3 ± 4.03	149 (74%)	17.8 ± 4.03
Thr/Ile	18 (25%)	15.1 ± 2.43	25 (19%)	14.8 ± 2.90	43 (21%)	14.9 ± 2.68
Ile/Ile	2 (2%)	14.3 ± 2.28	8 (7%)	12.4 ± 4.27	10 (5%)	13.8 ± 3.94
Allele Thr	124 (84.9%)		217 (84.1%)		341 (84.4%)	
Allele Ile	22 (15.1%)		41 (15.9%)		63 (15.6%)	

Difference at Thr147Ala between males and females, $*P < 0.05$, $**P < 0.01$.
Difference in plasma TAFI level between genotypes, $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.005$, $^{\#}P < 0.001$.

Table 2a. Age distribution of the total 202 subjects and plasma TAFI levels

		–20	21–30	31–40	41–50	–50	51–	51–60	61–70	71–80	81–	Total
N	Male	1	17	17	13	48	25	9	9	3	4	73
Plasma TAFI level		12.5	18.2	17.1	18.7	17.8	17.8	19.6	16.9	15.3	17.4	17.8
(mean ± SD)			± 3.70	± 4.00	± 3.89	± 3.71	± 4.03	± 4.40	± 4.30	± 2.91	± 2.13	± 3.90
	Female	8 *	28	21 **	30	87	* 52	18	14	4	6	129 *
		16.1	14.3	16.4	15.6	15.4	19.0	19.1	20.3	18.9	15.6	16.6
		± 5.36	± 3.10	± 2.91	± 3.27	± 3.41	± 4.17	± 3.91	± 4.35	± 3.77	± 3.71	± 4.02
	Total	9	45	38	43	135	77	27	23	7	10	202
		15.7	15.8	16.7	16.5	16.3	18.5	19.3	19.0	17.4	16.3	17.0
		± 5.15	± 3.81	± 3.41	± 3.71	± 3.75	± 4.13	± 4.01	± 4.56	± 3.69	± 3.17	± 4.01

$*P < 0.001$, $**P = 0.01$.

than 50. In subjects over 70, plasma TAFI levels of both sexes were reduced. Women under 50 years of age had a plasma TAFI level that was lower than that of all men and women older than 50. These findings are presented in Table 2b.

Plasma TAFI Level with Age for Each Genotype

As previously reported, plasma TAFI levels and activity are influenced by single nucleotide polymorphisms (SNPs) (20). In a simple comparison of Thr/Ala-147 and Thr/Ile-325 alleles of males and females, only Thr325Thr females had a significantly lower plasma TAFI level than males (P=0.019; Table 1b). In comparing genotypes, Ala147Ala and Ile325Ile were associated with the lowest levels in both males and females and this association was statistically significant.

Table 2b provides an interesting comparison of SNP genotypes of those younger and older than 50 years of age. Only males showed a genotypic difference.

The reason for the difference in males is not clear and it should be pointed out that significance was found only at 147 (Thr/Thr vs. Ala/Ala) in the over-50 group and at 325 (Thr/Thr vs. Thr/Ile) in the under-50 group (data not shown).

With females, there was a distinct difference between the younger and older than 50 groups with respect to positions 147 (Thr/Ala, Ala/Ala) and 325 (Thr/Thr). Table 2c shows P values for the genotypes of females under and over age 50.

Discussion

Genetic Distribution of All Subjects and of Normal Subjects Only

As mentioned in "Results," the genotype distribution

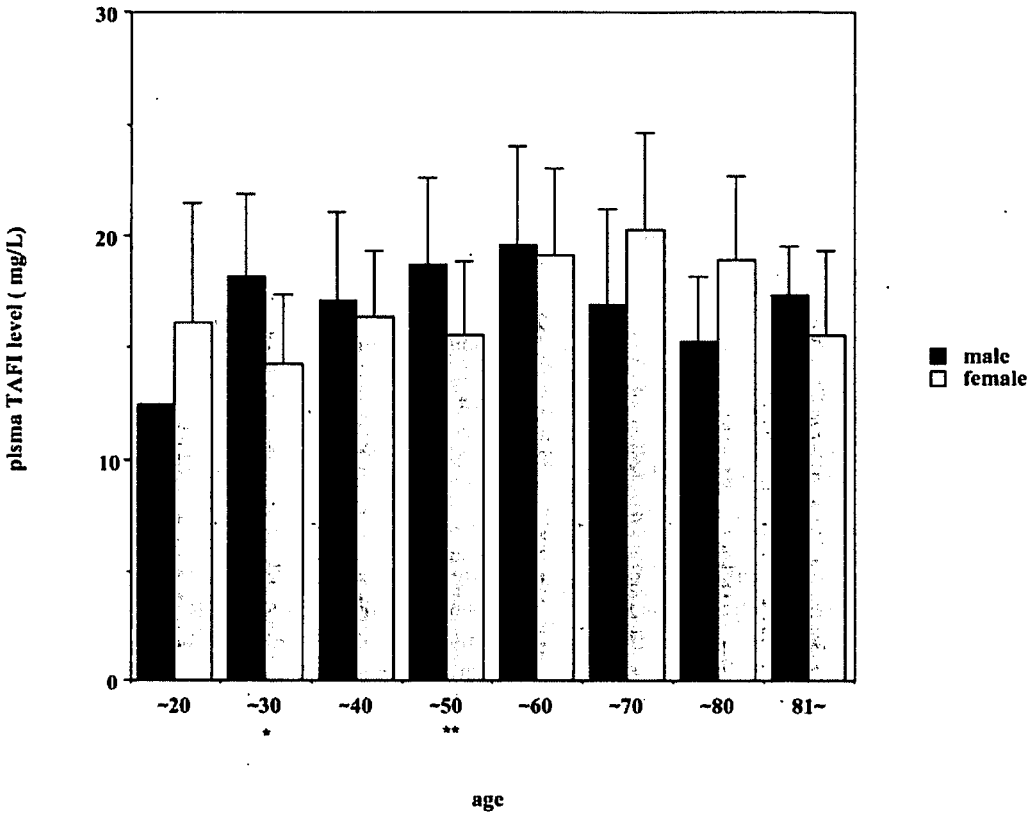


Fig. 1. Plasma TAFI level for each age group of males and females. The graph shows age group means (columns) and SD (bars). $*P < 0.05$, $** P < 0.005$ male versus female.

Table 2b. Genotype distribution of the 202 normal subjects and plasma TAFI levels before and after 50 years of age

	Males		Females		Total	
	N; mean ± SD (mg/liter)		*N*; mean ± SD (mg/liter)		*N*; mean ± SD (mg/liter)	
	-50	51-	-50	51-	-50	51-
Thr147Ala						
Thr/Thr	3; 21.4 ± 4.46	3; 22.0 ± 5.87	8; 18.5 ± 2.25	3; 22.2 ± 5.06	11; 19.3 ± 3.06	6; 22.1 ± 4.90
Thr/Ala	33; 18.0 ± 3.81	11; 18.5 ± 2.79	33; 16.6 ± 3.66	17; 19.9 ± 4.67	66; 17.3 ± 3.78	28; 19.4 ± 4.04
Ala/Ala	12; 16.4 ± 3.53	11; 15.9 ± 3.84	46; 14.0 ± 2.70	22; 17.8 ± 3.34	58; 14.5 ± 3.02	33; 17.2 ± 3.56
Allele Thr	39 (40.6%)	17 (34.0%)	49 (28.2%)	23 (27.4%)	88 (32.6%)	40 (29.9%)
Allele Ala	57 (59.4%)	33 (66.0%)	125 (71.8%)	61 (72.6%)	182 (67.4%)	94 (70.1%)
Thr325Ile						
Thr/Thr	36; 18.9 ± 3.68	17; 18.9 ± 4.28	63; 16.0 ± 3.35	33; 19.6 ± 4.20	99; 17.1 ± 3.72	50; 19.4 ± 4.20
Thr/Ile	11; 14.6 ± 2.68	7; 15.8 ± 1.92	19; 14.2 ± 2.83	6; 16.5 ± 2.53	30; 14.4 ± 2.74	13; 16.1 ± 2.16
Ile/Ile	1; 12.7	1; 15.9	5; 12.1 ± 3.71	3; 16.3 ± 4.79	6; 12.7 ± 3.66	4; 15.4 ± 4.31
Allele Thr	83 (86.5%)	41 (82.0%)	145 (83.3%)	72 (85.7%)	228 (84.4%)	113 (84.3%)
Allele Ile	13 (13.5%)	9 (18.0%)	29 (16.7%)	12 (14.3%)	42 (15.6%)	21 (15.7%)

for each group did not deviate significantly from HWE. However, as shown in Table 1a and b, the difference between males and females was due to the male Ala/Ala-147 volunteers. Compared with our previous group of population-based aged controls (1) the *P* value in this report was 0.042 male Thr147Ala alleles in males. In this study, 73 males among the 202 normal subjects were aged 45.4 ± 18.27 while the remaining ranged from 20–93, and all were from central Japan. Our previous samples (1) were from an 80.5 ± 8.0 year-old population from western Japan. This gap in age was not considered significant with respect to Thr147Ala alleles. In fact, as seen in Table 2b, the genotype distributions for those in the before and after

Table 2c. Statistical representation of the difference in the plasma TAFI levels of females with each genotype before and after 50 years of age

Thr147Ala		*P* value –50			*P* value 51–		*P* value –50 vs. 51–
	Thr/Thr	Thr/Ala	Ala/Ala	Thr/Thr	Thr/Ala	Ala/Ala	
Thr/Thr		-	-		-	-	0.11
Thr/Ala	0.17		-	0.45		-	0.0085
Ala/Ala	0.00005	0.005		0.05	0.11		0.003
Thr325Ile		*P* value –50			*P* value 51–		*P* value –50 vs. 51–
	Thr/Thr	Thr/Ile	Ile/Ile	Thr/Thr	Thr/Ile	Ile/Ile	
Thr/Thr		-	-		-	-	0.00001
Thr/Ile	0.037		-	0.090		-	0.089
Ile/Ile	0.015	0.18		0.20	0.93		0.210

$P < 0.05$, $P < 0.005$.

age 50 groups were not significantly different. Accordingly, any genotypic gap relative to Thr147Ala in normal males would reflect a regional difference. However, for males and females in general, the Thr147Ala distributions presented previously by us (1) and others (8) were not significantly different from that of the present report.

In contrast, in the present study as in our previous report (1), findings regarding Thr325Ile, allele frequencies differed significantly from those of other investigators (8, 24).

This difference in allele frequencies at position 325 might simply be due to a racial difference between Caucasians and Japanese. In addition, some of the samples in our previous report were from subjects older than 80 years (1), and it is possible that the deviation observed in that study might have reflected the longer life span of our patients.

However, the average age of subjects in our previous report was around 60 years, while in other studies, it was around 50 (8, 21). The genotypic distribution of our Japanese subjects would also be expected to differ from that of Caucasians.

Plasma TAFI Level with Age for Each Genotype

It is obvious that plasma TAFI levels and activity are influenced by SNPs (20, 38). Although it cannot be seen clearly in Fig. 2, Table 1b shows a significant correlation between the plasma TAFI level and genotype. Among Thr147Ala and Thr325Ile polymorphisms, subjects with the Thr/Thr type had the highest TAFI level. On comparing males and females, male levels seemed higher but there was no statistical significance to this difference unless Thr/Thr at Thr325Ile was included. Our anti-proCPR-specific (anti-TAFI) monoclonal ELISA system also detected SNP differences.

In terms of age dependence, the TAFI level was down-regulated in the group of younger females, as seen in Fig. 1. In addition, this tendency was noted in each genetic type (Fig. 2b and d).

To clarify the data, the female plasma TAFI level was analyzed according to decade, and a statistically significant difference was detected between the 51–70 vs. 21–50 groups (Table 2b and c). The TAFI antigen level could be influenced by hormonal status and may reflect menopausal changes, as reported by Chetaille et al. (12).

All data were separated according to genotype into before and after age 50 groups and no significant difference was found in their genetic distribution. However, in each SNP group, the TAFI plasma level of the younger subjects (younger than age 50) differed significantly.

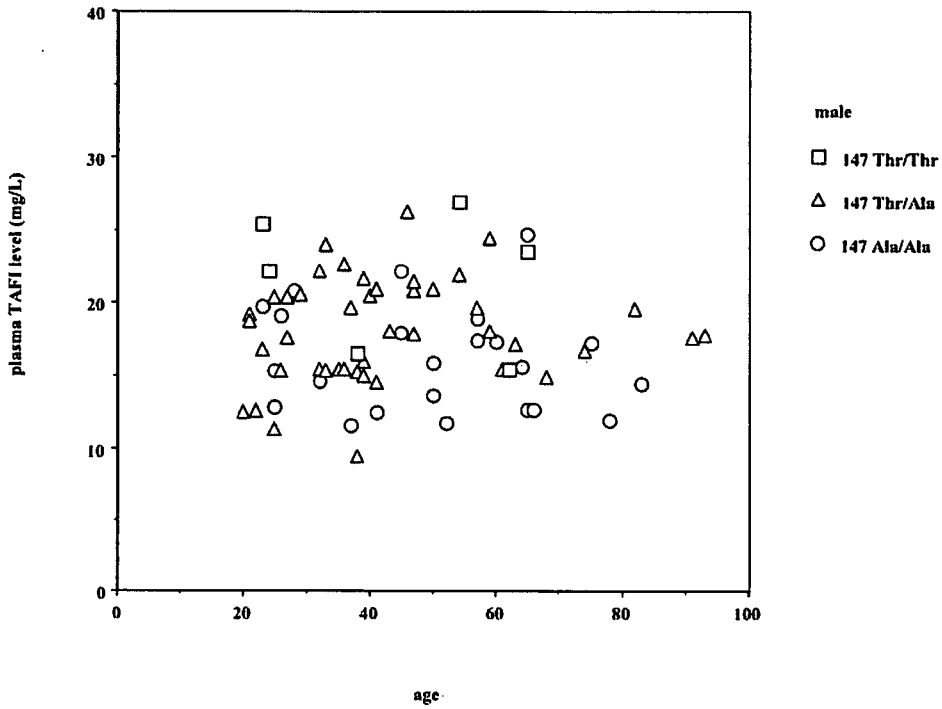
In subjects with the Thr/Ala and Ala/Ala alleles of Thr147Ala, the proCPR levels in the before versus the after age 50 groups were significantly different (Table 2c). For those who were 51 or older, the *P* value for Thr/Thr versus Ala/Ala was only 0.05. On the other hand, in those under 50, the Ala/Ala group deviated significantly from the Thr/Thr and Thr/Ala groups with regard to the plasma TAFI level (Table 2c).

This tendency was also observed with Thr325Ile. In this study, the Thr/Thr deviation was strongly significant. There was also a significant difference between this polymorphism and Thr/Ile and Ile/Ile, but only in the under-50 group.

In conclusion, the Ala/Ala at 147 and Ile/Ile at 325 group showed a tendency toward low levels of plasma proCPR (TAFI). However, in females, this tendency disappeared at around 50 years of age, suggesting a strong association with the onset of menopause.

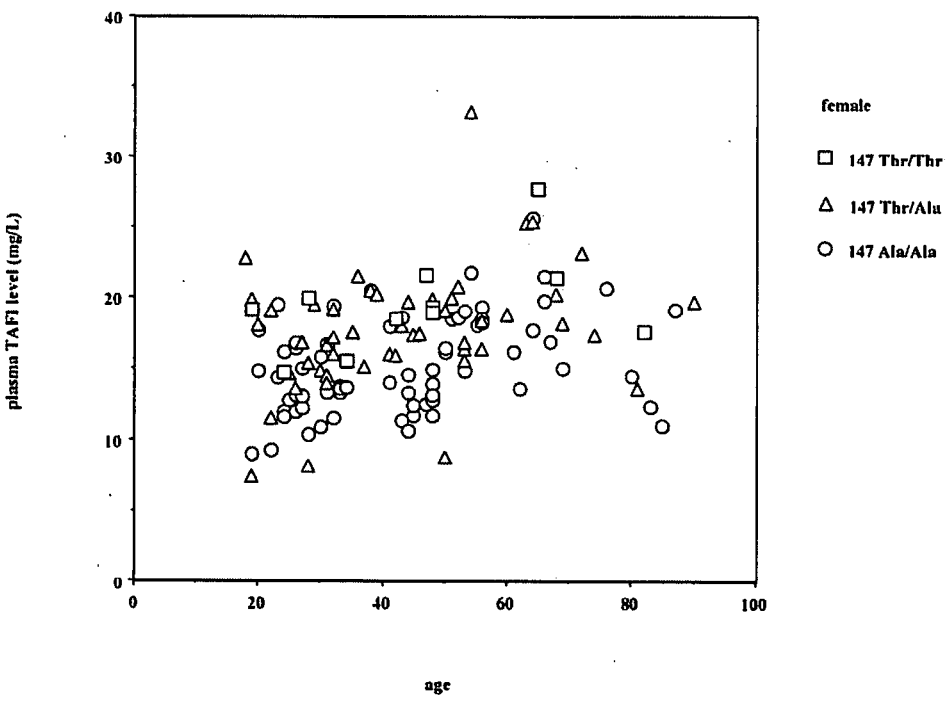
Before menopause, normal women experience a

(a)

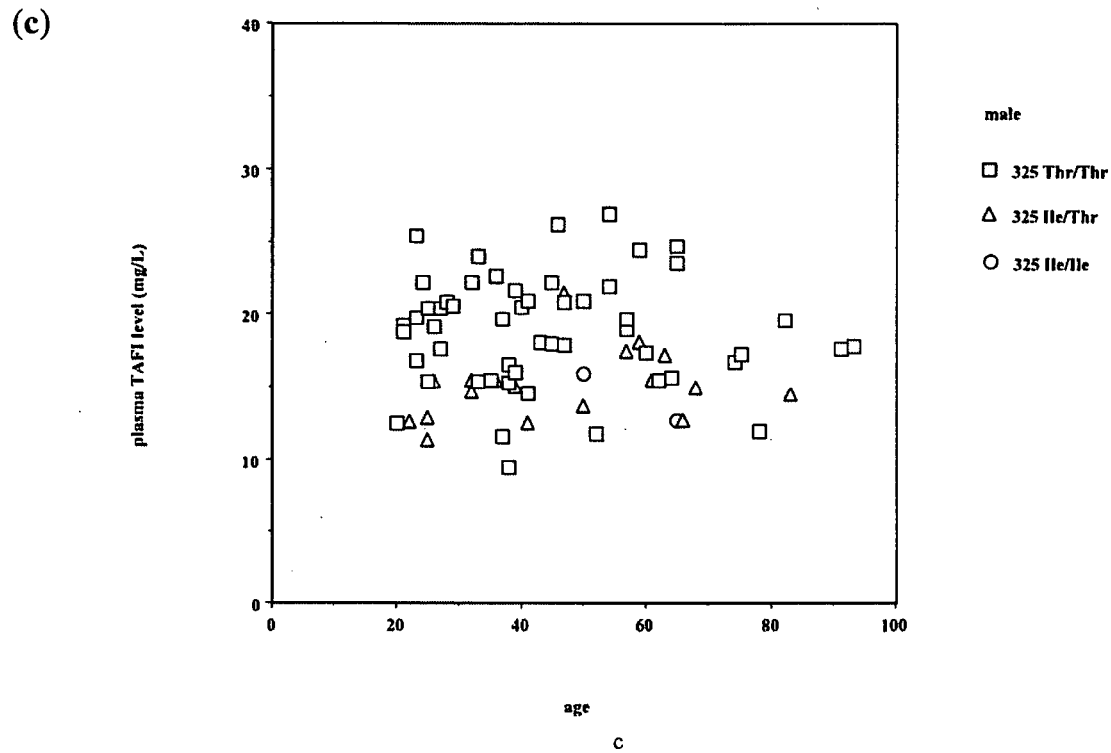

plasma TAFI level (mg/L)
40
30
20
10
0
0
20
40
60
80
100
age
male
147 Thr/Thr
147 Thr/Ala
147 Ala/Ala

a

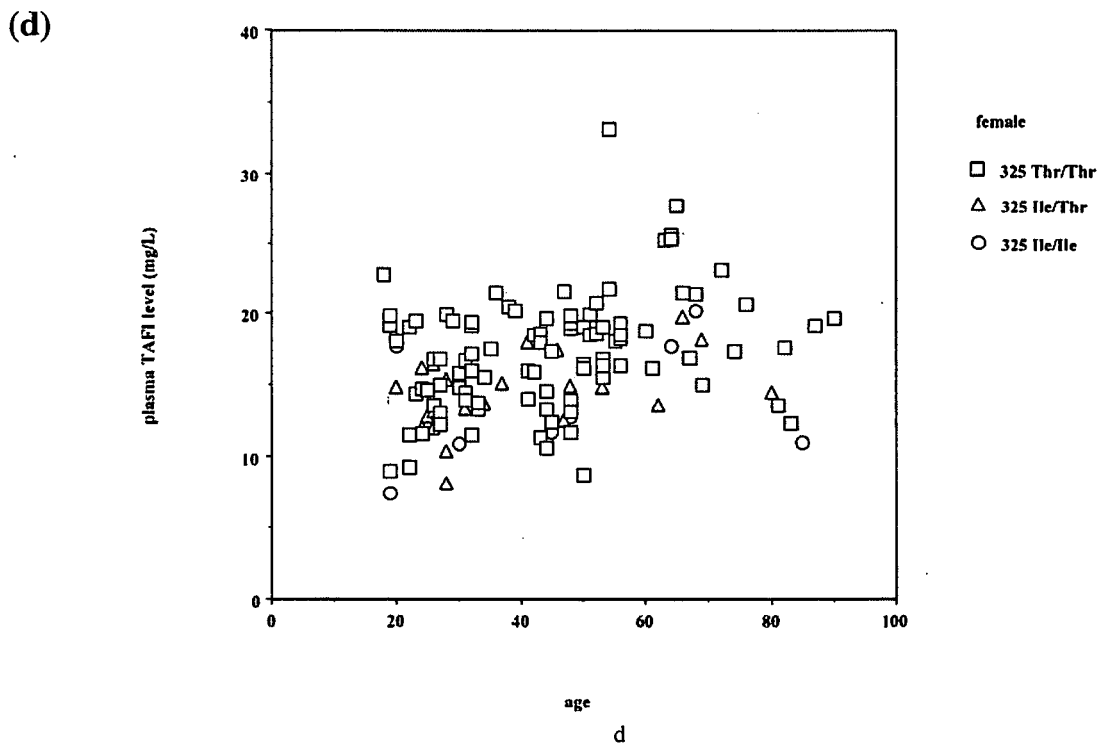
(b)


plasma TAFI level (mg/L)
40
30
20
10
0
0
20
40
60
80
100
age
female
147 Thr/Thr
147 Thr/Ala
147 Ala/Ala

b



c



d

Fig. 2. Plasma TAFI level in males and females correlated with Thr147Ala and Thr325Ile genotypes. a: male Thr147Ala, b: female Thr147Ala. □ 147 Thr/Thr, △ 147 Thr/Ala, ○ 147 Ala/Ala. c: male Thr325Ile, d: female Thr325Ile. □ 325 Thr/Thr, △ 325 Ile/Thr, ○ 325 Ile/Ile. Each spot represents an individual.

monthly estradiol/progesterone menstrual cycle. Estradiol might be a particular candidate for reducing TAFI levels since oral estradiol/trimegestone replacement reduces TAFI production in early postmenopausal women (28) and 3 months of raloxifene treatment was associated with a significant decrease in the plasma TAFI antigen concentration (27). However, another study dismissed the short-term effects of estrogen, tamoxifen and raloxifene on the plasma TAFI level (13). Moreover, the TAFI level changes throughout a normal pregnancy, suggesting that it must correspond to hormonal fluctuations occurring during gestation.

Little is known of the biochemical mechanism underlying the hormonal effect on TAFI levels, especially in individuals with Ala/Ala at 147 or Thr/Thr at 325. However, as with the immunological response, lipopolysaccharide is likely a trigger (31). Molecular analysis of the human TAFI promoter region shows that the CCAAT/enhancer-binding protein (C/EBP)-binding site occupies positions −53 and −40 (5) and the functional glucocorticoid response element (GRE) occupies positions −92 and −78 (6). This suggests that TAFI is not only an acute phase protein but also regulates steroid hormones.

Comparison of Plasma TAFI Levels and Other Biochemical Data

Plasma TAFI is generated in liver (14) and platelets (25), and is up-regulated in acute inflammatory states (31). The concentration of TM is an important factor in the regulation of TAFI activation as well as in the regulation of fibrinolysis. High TM concentrations result in down-regulation of fibrinolysis, whereas low TM concentrations result in up-regulation (26). The thrombin-thrombomodulin complex (T/TM), rather than free thrombin, is the most likely physiological activator of TAFI (3).

However, under normal conditions, TAFI levels showed no correlation with platelets, with the liver enzymes AST and ALT, with the WBC count as an inflammatory marker, or with TM as a regulator of TAFI activation (data not shown).

In previous reports, the risk of acute coronary artery disease was associated with a disturbance in functional thrombin activatable fibrinolysis inhibitor plasma levels (30) as was the risk of deep vein thrombosis (39). Obstructive vascular disease as well as inflammatory states, burns and septic injuries induce profound changes in the coagulation status. In a rat model of burn and septic injury, TAFI levels increased significantly at 24- and 72-hr time points following burn, caecal ligation and puncture (CLP), and burn + CLP (29). In addition, TAFI mRNA was found to be up-regulated in an LPS model (31). In contrast, Watanabe et al. measured plasma levels of TAFI antigen and activity in patients with disseminated intravascular coagulation (DIC) to examine the relationship between hypofibrinolysis and the pathogenesis of DIC and found that TAFI levels and activity in plasma were significantly low in these patients (40).

This was not a multi-center project, and our samples were limited to the patients and volunteers of a single hospital. Nevertheless, we were able to establish the normal plasma proCPR (TAFI) level taking age and Thr147Ala and Thr325Ile status into account. Because of variation in the geographical origin of our subjects, there was some deviation at Thr147Ala. However, considering genetics and age, a level of about 8–25 mg/liter was established as normal and not to reflect the influence of other factors. In future studies using these data, we will analyze the relationship between plasma proCPR (TAFI) levels and several thrombotic and inflammatory conditions.

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