Table 2 Subject characteristics

	All (n = *630*)	*Male* (n = *340*)	*Female* (n = *290*)	P-*value*
Age (years)	64.6±10.6	63.3±11.3	66.0±9.6	0.0015
Height (cm)	160.0±8.7	165.8±6.4	153.1±5.5	<0.0001
Weight (kg)	62.9±11.6	68.5±10.6	56.4±9.1	<0.0001
Heart rate (b.p.m.)	64.0±10.7	62.0±9.4	66.2±11.8	<0.0001
Systolic blood pressure (mm Hg)	138.8±17.1	137.0±15.8	140.9±18.3	0.0042
Diastolic blood pressure (mm Hg)	82.7±10.3	83.2±10.2	82.1±10.5	0.1799
Mean IMT (mm)	0.83±0.16	0.83±0.16	0.84±0.17	0.4634
Plaque score	3.13±4.76	3.57±5.18	2.61±4.17	0.0131
baPWV (cm/s)	1786.2±309.1	1755.7±297.7	1822.0±318.8	0.0071
ABI	1.12±0.08	1.13±0.09	1.11±0.07	0.0018
CRP (mg/dl)	0.15±0.28	0.17±0.20	0.14±0.30	0.1728
HbA_{1c} (%)	5.63±0.80	5.66±0.77	5.58±0.83	0.2259
Total cholesterol (mg/dl)	203.0±35.2	196.7±30.4	210.4±39.0	<0.0001
Triglyceride (mg/dl)	138.3±125.3	152.4±149.7	121.5±85.3	0.0020
HDL-cholesterol (mg/dl)	52.7±15.2	48.7±13.0	57.4±16.3	<0.0001
Smoking (current/past/never)	69/211/339	59/183/89	10/28/250	<0.0001
Anti-hypertensive medication (%)	570/630 (90.5%)	308/340 (90.6%)	262/290 (90.3%)	0.9174

Abbreviations: ABI, ankle brachial index; baPWV, brachial-ankle pulse wave velocity; CRP, C-reactive protein; HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; Mean IMT, mean intima-media thickness.
Values are expressed as the means±s.d. *P* ; Student's *t*-test (male vs female).

Table 3a Comparison between SNPs of ET-1 genes and baPWV in male subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*baPWV (cm/s)*	P-*dominamt*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	251	1763.8±301.0	0.4250	0.6509	0.7682
			3A4A	81	1730.3±291.5			
			4A4A	5	1795.3±271.3			
	rs2070699	T/G	TT	104	1768.1±341.2	0.6029	0.3509	0.2902
			TG	158	1731.8±265.6			
			GG	76	1787.3±297.1			
	rs5370	G(Lys)/T(Asn)	GG	182	1759.6±308.5	0.8425	0.8318	0.5438
			GT	134	1758.8±286.2			
			TT	23	1720.2±284.5			
EDNRA	rs5333	T/C	TT	182	1746.8±307.0	0.5958	0.4479	0.2086
			TC	130	1752.5±269.4			
			CC	23	1830.1±369.9			
EDNRB	rs 5351	A/G	AA	107	1706.6±285.1	0.0409 (0.4499)*	0.0004 (0.0044)*	0.0001 (0.0011)*
			AG	162	1736.1±277.7			
			GG	65	1882.2±332.7			
	rs3818416	G/T	GG	305	1759.9±301.1	0.2393	0.3593	0.2593
			GT	28	1708.0±260.2			
			TT	3	1560.5±241.2			
ECE1	rs212526	C/T	CC	247	1746.9±294.9	0.4798	0.7583	0.9557
			CT	82	1775.1±298.4			
			TT	7	1747.6±415.2			
	rs212528	T/C	TT	198	1724.6±292.4	0.0311 (0.3421)*	0.0246	0.3099
			TC	122	1810.8±298.3			
			CC	16	1679.9±308.2			
	rs213045	G/T	GG	102	1732.0±282.7	0.3865	0.3293	0.3737
			GT	174	1776.5±305.3			
			TT	59	1722.0±301.3			
	rs2038089	A/G	AA	153	1773.4±300.4	0.3051	0.0821	0.0262 (0.2882)*
			AG	138	1764.3±304.3			
			GG	43	1661.1±253.9			
ECE2	rs2272471	C/T	CC	94	1778.0±303.1	0.3573	0.6116	0.9717
			CT	164	1739.8±282.3			
			TT	76	1755.1±324.4			

Abbreviations: baPWV, brachial-ankle pulse wave velocity; SNPs, single nucleotide polymorphisms.
P-value (dominant), major vs hetero+minor; *P*-value (additive), major vs heterozygote vs minor; *P*-value (recessive), minor+hetero vs major.
*Bonferroni correction (× 11).

Table 3b Comparisons between ET-1 gene SNPs and baPWV in female subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*baPWV (cm/s)*	P-*dominant*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	198	1831.0±329.4	0.4510	0.7278	0.9152
			3A4A	84	1798.0±305.9			
			4A4A	5	1836.6±199.9			
	rs2070699	T/G	TT	80	1845.0±367.1	0.4673	0.7631	0.7183
			TG	139	1816.2±305.3			
			GG	69	1810.8±289.1			
	rs5370	G (Lys)/T(Asn)	GG	147	1843.9±321.8	0.2519	0.4711	0.5298
			GT	116	1795.2±316.4			
			TT	26	1825.8±319.9			
EDNRA	rs5333	T/C	TT	153	1796.2±306.1	0.1163	0.1601	0.5298
			TC	116	1867.4±331.3			
			CC	17	1776.6±342.9			
EDNRB	rs 5351	A/G	AA	85	1859.2±315.1	0.2257	0.3921	0.3211
			AG	145	1817.9±339.8			
			GG	56	1785.8±268.3			
	rs3818416	G/T	GG	255	1822.4±325.2	0.8168	0.3676	(–)
			GT	29	1821.6±270.6			
			TT	1	2277.0			
ECE1	rs212526	C/T	CC	208	1827.0±308.5	0.7909	0.4074	0.1873
			CT	67	1835.0±360.3			
			TT	11	1698.1±257.9			
	rs212528	T/C	TT	184	1833.1±320.8	0.5150	0.4206	0.3855
			TC	86	1791.7±307.6			
			CC	16	1891.4±371.4			
	rs213045	G/T	GG	93	1834.6±369.1	0.6899	0.4138	0.2837
			GT	142	1801.0±281.4			
			TT	50	1867.8±326.9			
	rs2038089	A/G	AA	124	1821.2±322.9	0.8902	0.9691	0.8109
			AG	131	1824.3±321.9			
			GG	24	1839.2±323.7			
ECE2	rs2272471	C/T	CC	73	1795.8±343.4	0.3612	0.5926	0.4611
			CT	144	1828.5±314.6			
			TT	68	1850.3±304.4			

Abbreviations: baPWV, brachial-ankle pulse wave velocity; SNPs, single nucleotide polymorphisms.
P-value (dominant); major vs hetero+minor, *P*-value (additive); major vs heterozygote vs minor, *P*-value (recessive); minor+hetero vs major.

Screening of genetic variations in EDN1 EDNRA, EDNRB, ECE1 and ECE2

We isolated genomic DNA from the peripheral blood leukocytes of 630 subjects and directly sequenced the entire coding region of the endothelin-1 gene (*EDN1*). The results of the *EDN1* screening are shown in Table 1. Finally, we selected three SNPs in the *EDN1*. We selected SNPs of the endothelin type A receptor gene (*EDNRA* rs5333), endothelin type B receptor gene (*EDNRB* rs5351, rs3818416), endothelin converting enzyme-1 gene (*ECE1* rs212526, rs212528, rs213045, rs2038089) and endothelin converting enzyme-2 gene (*ECE2* rs2272471) from a public database (dbSNP http://www.ncbi.nlm.nih.gov/SNP/). SNPs with a minor allele frequency of greater than 5% were genotyped using the TaqMan-PCR method described previously.[16] The representative SNPs were genotyped when they were linkage disequilibrium (LD: r^2 over 0.5). The LD was calculated between each SNP. The primers and probes used in the TaqMan-PCR system are available upon request.

Statistical analysis

Values are expressed as means ±s.d. and were analyzed using a Student's *t*-test and a χ^2-test where appropriate. Hardy–Weinberg equilibrium was assessed by χ^2analysis, and we considered *P*-values less than 0.05 to be statistically significant. The levels of the *P*-values were adjusted by Bonferroni correction). The LD between each SNP was checked using Haploview version 4 (http://www.broad.mit.edu/mpg/haploview/). The association of genotypes with blood pressure, IMT and PS of carotid arteries and baPWV was examined by simple regression analysis and then investigated using a logistic regression model that adjusted for confounding factors. The distribution of plaque score (PS) was not normal, so we compared the prevalence of severe PS ($\geqslant$10.1)[17] for each allele. All statistical analyses were performed using Stat-View version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Patient Characteristics and the Correlation between baPWV and Clinical Parameters

The characteristics of the subjects at baseline are summarized in Table 2. Significant differences were apparent between men and women in age, height, weight, heart rate (HR), systolic blood pressure (SBP), plaque score (PS), baPWV and ABI and lipid

Table 4a Comparisons between ET-1 gene SNPs and mean IMT in male subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*Mean IMT*	P-*dominant*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	250	0.824±0.160	0.7936	0.1199	0.0400 (0.4400)*
			3A4A	81	0.821±0.152			
			4A4A	5	0.970±0.148			
	rs2070699	T/G	TT	104	0.815±0.143	0.3957	0.6548	0.5235
			TG	157	0.829±0.163			
			GG	76	0.837±0.170			
	rs5370	G (Lys)/T(Asn)	GG	181	0.832±0.154	0.4286	0.5953	0.7007
			GT	134	0.815±0.162			
			TT	23	0.838±0.174			
EDNRA	rs5333	T/C	TT	181	0.821±0.160	0.4330	0.0023 (0.0253)*	0.0005 (0.0055)*
			TC	130	0.816±0.146			
			CC	23	0.937±0.179			
EDNRB	rs 5351	A/G	AA	107	0.830±0.164	0.8131	0.0104 (0.1144)*	0.0059 (0.0649)*
			AG	161	0.805±0.147			
			GG	65	0.875±0.168			
	rs3818416	G/T	GG	304	0.828±0.161	0.5352	0.7307	0.5119
			GT	28	0.814±0.130			
			TT	3	0.767±0.161			
ECE1	rs212526	C/T	CC	246	0.832±0.161	0.3202	0.5493	0.4919
			CT	82	0.786±0.157			
			TT	7	0.814±0.152			
	rs212528	T/C	TT	198	0.826±0.156	0.9406	0.9714	0.8410
			TC	121	0.828±0.165			
			CC	16	0.819±0.153			
	rs213045	G/T	GG	102	0.831±0.179	0.7299	0.6596	0.3631
			GT	174	0.829±0.154			
			TT	58	0.809±0.134			
	rs2038089	A/G	AA	152	0.830±0.161	0.7842	0.8860	0.6774
			AG	138	0.828±0.144			
			GG	43	0.816±0.195			
ECE2	rs2272471	C/T	CC	93	0.820±0.160	0.6244	0.8500	0.9127
			CT	164	0.832±0.161			
			TT	76	0.826±0.153			

Abbreviations: SNPs, single nucleotide polymorphisms; IMT, intima-media thickness.
P-value (dominant), major vs hetero+minor; *P*-value (additive), major vs heterozygote vs minor; *P*-value (recessive), minor+hetero vs major.
*Bonferroni correction (× 11).

profiles. Almost all subjects were treated with antihypertensive agents such that the ratio of treated patients did not differ between males and females.

We analyzed the correlations between baPWV, mean IMT, max IMT, PS of carotid arteries and the clinical parameters of male and female patients. BaPWV significantly correlated with age, height, weight, SBP, DBP, mean BP, HR and HbA_{1c}. In contrast, baPWV was not associated with serum creatinine, C-reactive protein, or ABI. The mean IMT and PS of carotid arteries significantly correlated with age, height, HbA_{1c} and HDL-Chol. All indices of atherosclerosis strongly associated with age, height and BP. Among these indices, IMT and PS showed weaker association with weight and BP than baPWV.

Correlation between baPWV and SNPs in ET-1 family genes

We studied 11 SNPs in total, including three of *EDN1*, one of *EDNRA*, two of *EDNRB*, four of *ECE1* and one of *ECE2*. We found no tight LD between the 11 analyzed SNPs. We analyzed the association of baPWV with ET-1 SNPs in all subjects, both male and female. As shown in Table 3a, we detected significant differences in baPWV in comparing additive, dominant, or recessive models in *EDNRB*-rs5351 (exon 6), *ECE1*- rs212528 (intron 3) and rs2038089 (intron 17) in male subjects. Finally, only *EDNRB* -rs5351 positively associated with baPWV after performing a Bonferroni correction. No SNPs were significantly associated with baPWV in female subjects (Table 3b).

Mean IMT, max IMT, plaque score of carotid arteries and ET-1 SNPs

The results of comparing additive, dominant, or recessive models for mean IMT in each SNP are shown in Tables 4a and 4b. Only *EDNRA*-rs5333 positively associated with mean IMT after performing a Bonferroni correction, and this association was only apparent in male subjects. With regard to max-IMT, *EDNRA*-rs5333, *EDNRB* -rs5351 and *ECE1*- rs2038089 showed a positive association, but the association was not significant after Bonferroni correction (data not shown). In comparing the prevalence of severe PS ($\geqslant$10.1) for each allele, no

Table 4b Comparisons between ET-1 gene SNPs and mean IMT in female subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*Mean IMT*	P-*dominant*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	196	0.841±0.175	0.6400	0.1625	0.0706
			3A4A	83	0.828±0.152			
			4A4A	5	0.700±0.079			
	rs2070699	T/G	TT	79	0.839±0.170	0.8018	0.1429	0.0546
			TG	137	0.849±0.174			
			GG	69	0.801±0.152			
	rs5370	G (Lys)/T(Asn)	GG	144	0.835±0.168	0.9802	0.3176	0.1531
			GT	116	0.846±0.172			
			TT	26	0.835±0.168			
EDNRA	rs5333	T/C	TT	152	0.826±0.156	0.3386	0.0463 (0.5093)*	0.1527
			TC	115	0.858±0.182			
			CC	17	0.759±0.139			
EDNRB	rs 5351	A/G	AA	84	0.836±0.152	0.9740	0.9909	0.9095
			AG	144	0.834±0.171			
			GG	56	0.837±0.186			
	rs3818416	G/T	GG	253	0.830±0.167	0.1547	0.2753	(–)
			GT	29	0.872±0.178			
			TT	1	1.000			
ECE1	rs212526	C/T	CC	206	0.834±0.168	0.9034	0.6696	0.4238
			CT	67	0.844±0.169			
			TT	11	0.795±0.159			
	rs212528	T/C	TT	183	0.831±0.158	0.5586	0.5997	0.5508
			TC	86	0.849±0.182			
			CC	15	0.810±0.205			
	rs213045	G/T	GG	92	0.826±0.162	0.5215	0.7194	0.4975
			GT	141	0.836±0.162			
			TT	50	0.850±0.196			
	rs2038089	A/G	AA	126	0.818±0.166	0.1171	0.2132	0.2300
			AG	130	0.845±0.170			
			GG	24	0.875±0.174			
ECE2	rs2272471	C/T	CC	71	0.847±0.186	0.5061	0.0360 (0.3960)*	0.0327 (0.3597)*
			CT	144	0.812±0.154			
			TT	68	0.874±0.171			

Abbreviations: SNPs, single nucleotide polymorphisms; IMT, intima-media thickness.
P-value (dominant), major vs hetero+minor; *P*-value (additive), major vs heterozygote vs minor, *P*-value (recessive), minor+hetero vs major.
*Bonferroni correction (× 11).

SNPs were positive in either male or female subjects after Bonferroni correction (Table 5).

Association of SNPs in ET-1 family genes with severe atherosclerosis

We have compared the atherosclerosis parameters and the background of each genotype of *EDNRA*-rs5333, *EDNRB*-rs5351, *ECE1*-rs212528 and rs2038089. These four SNPs showed significant association with atherosclerotic indices, including baPWV, PS and IMT. They had no association with atherosclerotic risk factors, such as HbA1c, TG, HDL-chol, except *EDNRA*-rs5333 which showed association with TG.

We divided the male subjects in three ways: a rapid or slow group based on the average baPWV (rapid: $\geqslant$1756 cm/s, slow: $<$1756 cm/s), averaged mean-IMT (severe: $\geqslant$0.86 mm, mild: $<$0.86 mm) and a severe or mild atherosclerotic group using plaque scores (severe: $\geqslant$10.1, mild: $<$10.1). We performed logistic regression analysis on the progression of baPWV, mean-IMT and PS. Multiple logistic regression analysis indicated that GG in *EDNRB* -rs5351, with higher baPWV and PS, was an independent risk factor in male subjects (Tables 6a–c).

Discussion

The human ET-1 gene was cloned and sequenced in 1989 by Inoue *et al.*[18] Recent studies have examined the relationship between polymorphisms of ET-1 and BP. Tiret *et al.*[19] indicated that a G/T polymorphism with an amino acid substitution (Lys → Asn) at codon 198 in exon 5 of ET-1 was associated with BP in overweight Europeans, and similar results were obtained in Japanese subjects.[20,21]

In this study, we evaluated the association of 11 SNPs of ET-1 family genes with atherosclerosis in hypertensive patients. We found a significant correlation between baPWV and *EDNRB*-rs5351 and between mean IMT of carotid arteries and *EDNRA*-rs5333 in male, but not female hypertensive patients after Bonferroni correction; however, EDNRA-rs5333 was not significantly associated with severe IMT thickening after multiple logistic regression analysis. Thus, *EDNRB*-rs5351 was the most suscep-

Table 5 Genotype distribution among the subjects with severe (≥10.1) or mild atherosclerosis (by plaque scores)

				Male				*Female*			
Genes	*SNPs*	*Allele (major/minor)*	*Genotype*	*Mild*	*Severe*	χ^2	P-*value*	*Mild*	*Severe*	χ^2	P-*value*
EDN1	A201- (4A/3A)	3A/4A	3A3A	223	22	0.970	0.6156	184	11	2.668	0.2635
			3A4A	71	9			80	3		
			4A4A	4	1			4	1		
	rs2070699	T/G	TT	92	10	0.275	0.8714	74	4	1.660	0.4361
			TG	143	14			131	6		
			GG	64	8			63	6		
	rs5370	G (Lys)/T(Asn)	GG	161	18	0.082	0.9600	136	7	1.733	0.4205
			GT	120	12			109	7		
			TT	19	2			23	3		
EDNRA	rs5333	T/C	TT	160	19	0.906	0.6357	145	7	1.733	0.4204
			TC	116	10			106	8		
			CC	20	3			15	2		
EDNRB	rs 5351	A/G	AA	99	4	9.898	0.0071 (0.0781)*	79	4	2.740	0.2541
			AG	144	16			137	7		
			GG	52	12			50	6		
	rs3818416	G/T	GG	269	29	0.354	0.8376	237	15	0.105	0.9487
			GT	25	3			27	2		
			TT	3	0			1	0		
ECE1	rs212526	C/T	CC	217	23	0.171	0.9179	192	13	0.744	0.6894
			CT	74	8			63	4		
			TT	6	1			11	0		
	rs212528	T/C	TT	180	16	1.354	0.5082	171	11	1.102	0.5763
			TC	103	14			80	6		
			CC	14	2			15	0		
	rs213045	G/T	GG	92	8	1.447	0.4851	88	4	1.569	0.4564
			GT	154	16			130	11		
			TT	50	8			47	2		
	rs2038089	A/G	AA	126	23	9.901	0.0071 (0.0781)*	121	4	3.383	0.1843
			AG	130	7			119	11		
			GG	39	2			23	1		
ECE2	rs2272471	C/T	CC	82	7	4.258	0.1190	66	5	1.914	0.3840
			CT	150	13			137	6		
			TT	63	12			62	6		

Abbreviation: SNPs, single nucleotide polymorphisms.
Men and women were divided into three groups for each genotype.
*Bonferroni correction (× 11).

tible endothelin-related SNP associated with atherosclerosis in male hypertensives. With regard to the gender differences between baPWV and/or arteriosclerosis and ET-1 family gene polymorphisms, one possible explanation is that the effect of ET-1 on vasoconstriction and atherosclerosis may differ between males and females. Tatchum-Talom *et al.*[22] described the vasoconstrictive effect of ET-1 as much greater in male rats than in female rats. Alternatively, oestrogen may reduce the vasoconstriction induced by ET-1.[23] Our current findings indicate that there are differences in the progression of atherosclerotic changes among hypertensive patients that depend on the genotypes of ET-1 family genes. Therefore, our findings provide important information regarding the use of hypertensive agents. Hypertensive agents should perhaps be prescribed after taking the polymorphisms in specific patients into consideration.

Lajemi *et al.*[24] showed that the *EDNRA* -231A/G and *EDNRB* 30G/A gene polymorphisms influence PWV in women, and the *EDNRB* 30G/A genotype related to the level of radial artery parameters in men. They suggested that these genes were involved in arterial stiffness. Funalot *et al.*[25] showed that *ECE 1B* C338A and *EDN1* Lys198Asn work together to modulate BP levels in women. Of the three SNPs tested in this study, only *EDNRB*-rs5351 was associated with baPWV and PS. This may be because baPWV reveals a more functional change, while PS indicates a more structural change.

In this study, most patients were treated with antihypertensive agents, some of which might affect PWV either directly or indirectly. We did not have detailed information on the drugs being taken by each subject, which could be seen as a limitation on our study. However, it has been reported that evaluations of PWV for monitoring arterial stiffness and in developing risk assessment strategies for hypertensive patients are useful.[26]

It will be important to determine serum ET-1 levels in patients to examine whether the cause of differences in baPWV or carotid arteriosclerosis between genotypes is dependent on only the

Table 6a Logistic regression analysis of baPWV and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRB- rs5351 (AA-AG)	0.99	(0.544–1.804)	0.9747	—	—	—
(AA-GG)	2.353	(1.110–4.989)	0.0256	—	—	—
ECE1- rs212528 (TT-TC)	—	—	—	1.833	(1.058–3.176)	0.0307
(TT-CC)	—	—	—	0.541	(0.146–2.003)	0.3575
Age	1.105	(1.070–1.142)	<0.0001	1.11	(1.075–1.147)	<0.0001
Height	0.937	(0.889–0.986)	0.0132	0.936	(0.889–0.985)	0.0118
Weight	1.006	(0.975–1.038)	0.7156	1.002	(0.970–1.034)	0.9137
Mean BP	1.071	(1.042–1.101)	<0.0001	1.071	(1.042–1.100)	<0.0001
HR	1.022	(0.993–1.052)	0.1323	1.023	(0.995–1.052)	0.1032

Abbreviations: baPWV, brachial-ankle pulse wave velocity; BP, blood pressure; ET-1, endothelin-1; HR, heart rate.
The average baPWV of male patients was 1756 cm/s. Rapid group, ⩾baPWV 1756 cm/s; slow group, <1756 cm/s.

Table 6b Logistic regression analysis of plaque scores and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRB- rs5351 (AA-AG)	3.255	(0.898–11.802)	0.0725	—	—	—
(AA-GG)	5.017	(1.308–19.239)	0.0187	—	—	—
ECE1- rs2038089 (AA-AG)	—	—	—	0.334	(0.132–0.844)	0.0205
(AA-GG)	—	—	—	0.356	(0.076–1.666)	0.1895
Age	1.09	(1.032–1.152)	0.0020	1.097	(1.038–1.160)	0.0010
Height	0.984	(0.907–1.068)	0.7006	0.988	(0.912–1.071)	0.7748
Weight	0.962	(0.906–1.021)	0.2047	0.956	(0.901–1.014)	0.1333
DBP	1.031	(0.986–1.077)	0.1843	1.039	(0.994–1.086)	0.0911

Abbreviations: DBP, diastolic blood pressure; ET-1, endothelin-1.
The severe atherosclerotic group of male patients refers to PS⩾10.1. Rapid group, ⩾PS 10.1; slow group, <10.1.

Table 6c Logistic regression analysis of mean-IMT and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRA- rs5333 (TT-TC)	1.066	(0.655–1.737)	0.7966	—	—	—
(TT-CC)	2.328	(0.846–6.406)	0.1018	—	—	—
EDNRB- rs5351 (AA-AG)	—	—	—	0.770	(0.449–1.319)	0.3409
(AA-GG)	—	—	—	1.349	(0.686–2.653)	0.3861
Age	1.049	(1.022–1.076)	0.0003	1.052	(1.025–1.081)	0.0002
Height	1.008	(0.969–1.048)	0.6995	1.008	(0.969–1.049)	0.6853
DBP	0.989	(0.965–1.014)	0.3835	0.992	(0.967–1.017)	0.5110
HbA_{1c}	1.188	(0.872–1.619)	0.2735	1.270	(0.932–1.730)	0.1301
HDL-CHOL	0.976	(0.958–0.995)	0.0115	0.979	(0.959–0.996)	0.0150

Abbreviations: ET-1, endothelin-1; IMT, intima-media thickness.
The average mean-IMT in male patients was 0.86 mm. Severe group, ⩾mean-IMT 0.86 mm; mild group, <0.86 mm.

ET-1 level or on the interaction of several hormonal systems. The negative vascular effects of ET-1 may contribute to the pathogenesis of hypertension and its complications in black patients.[27] Unfortunately, we did not have enough data regarding serum ET-1 levels to analyze the relationship between serum levels and ET-1 family gene polymorphisms.

It is also important to examine the influence of menopause on atherosclerosis in female subjects. However, most female subjects in the present study were older than 60 years, so it was impossible to clarify the influence of menopause in this study.

Any synergetic effects of polymorphisms on baPWV and PS should also be evaluated. In male subjects, baPWV values were slower in those with TT than with TC+CC of *ECE1*-T/C-rs212528, and were also slower in those with AA than with AG+GG of *EDNRB*-A/G-rs5351. We therefore compared baPWVs of subjects with both TT of *ECE1*-rs212528 and AA of *EDNRB*-rs5351 to those of subjects with both CC of *ECE1*-rs212528 and GG of *EDNRB*-rs5351. However, we did not obtain a stronger correlation for combined gene types than single genotypes (data not shown). We obtained similar results by analyzing IMT and PS.

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What is known about this topic

- ET-1 is thought to play important roles in the development of atherosclerosis through endothelial dysfunction and proliferation of vascular smooth muscle cells.
- There have been various studies of the relationship between polymorphisms of ET-1 and BP.

What this study adds

- EDNRB-rs5351 in exon6 might contribute to the progression of atherosclerosis in male patients with EHT.
- In future, an evaluation of these polymorphisms may be valuable for the treatment of hypertensive and/or atherosclerotic patients.

Abbreviations: ET-1, endothelin-1; BP, blood pressure; EHT, essential hypertension.

References

1 Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y *et al.* A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**: 411–415.
2 Lerman A, Edwards BS, Hallett JW, Heublen DM, Sandberg SM, Burnett Jr JC *et al.* Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N Engl J Med* 1991; **325**: 997–1001.
3 Hirai Y, Adachi H, Fujita Y, Hiratsuka A, Enomoto M, Imaizumi T. Plasma endothelin-1 level is related to renal function and smoking status but not to blood pressure: an epidemiological study. *J Hypertens* 2004; **22**: 713–718.
4 Takase H, Moreau P, Lüschner TF. Endothelin receptor subtypes in small arteries. Studies with FR 139317 and bosentan. *Hypertension* 1995; **25**: 739–743.
5 Arai H, Hori S, Aramori I, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 1990; **348**: 730–732.
6 Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K *et al.* Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *J Clin Invest* 1993; **92**: 1367–1373.
7 Warner TD. Characterization of endothelin synthetic pathways and receptor subtypes: physiological and pathological implications. *Eur Heart J* 1993; **14** (suppl I): 42–47.
8 Fukuroda T, Fujikawa T, Ozaki S, ishikawa K, yano M, Nishikibe M. Clearance of circulating endothelin-1 by ETB receptors in rats. *Biochem Biophy Res Commun* 1994; **199**: 1461–1465.
9 Morris MJ, Cox HS, Lambert GW, Kaye DM, Jennings GL, Meredith IT *et al.* Region-specific neuropeptide Y overflows at rest and during sympathetic activation in humans. *Hypertension* 1997; **29**: 137–143.
10 Nohria A, Garrett L, Johnson W, Kinlay S, Ganz P, Creager MA. Endothelin-1 and vascular tone in subjects with atherogenic risk factors. *Hypertension* 2003; **42**: 43–47.
11 Maeda S, Miyauchi T, Iemitsu M, Sugawara J, Nagata Y, Goto K. Resistance exercise training reduces plasma endothelin-1 concentration in healthy young humans. *J Cardiovasc Pharmacol TM* 2004; **44** (suppl 1): S443–S446.
12 Blacher J, Asmar R, Djane S, London GM, Safer ME. Aortic pulse wave velocity as a marker of cardiovascular risk in hypertensive patients. *Hypertension* 1999; **33**: 1111–1117.
13 McEniery CM, Qasem A, Schmitt M, Avolio AP, Cockcroft JR, Wilkinson IB. Endothelin-1 regulates arterial pulse wave velocity *in vivo*. *J Am Coll Cardiol* 2003; **42**: 1975–1981.
14 Vuurmans TJL, Boer P, Koomans HA. Effects of endothelin-1 and endothelin-1 receptor blockade on cardiac output, aortic pressure, and pulse wave velocity in humans. *Hypertension* 2003; **41**: 1253–1258.
15 Takiuchi S, Mannami T, Miyata T, Kamide K, Tanaka C, Kokubo Y *et al.* Identification of 21 single nucleotide polymorphisms in human hepatocyte growth factor gene and association with blood pressure and carotid atherosclerosis in Japanese population. *Atherosclerosis* 2004; **173**: 301–307.
16 Tanaka C, Kamide K, Takiuchi S, Kawano Y, Miyata T. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003; **26**: 301–306.
17 Handa N, Matsumoto M, Maeda H, Hougaku H, Ogawa S, Fukunaga R *et al.* Ultrasonic evaluation of early carotid atherosclerosis. *Stroke* 1990; **21**: 1567–1572.
18 Inoue A, Yanagisawa M, Kimura S, Katsuya Y, Miyauchi T, Goto K *et al.* The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci USA* 1989; **86**: 2863–2867.
19 Tiret L, Poirier O, Hallet V, McDonagh TH, Morrison C, McMurray JJ *et al.* The Lys198Asn polymorphism in the endothelin-1 gene is associated with blood pressure in overweight people. *Hypertension* 1999; **33**: 1169–1174.
20 Asai T, Ohkubo T, Katsuya T, Higaki J, Fu Y, Fukuda M *et al.* Endothelin-1 gene variant associates with blood pressure in obese Japanese subjects. The Ohasama study. *Hypertension* 2001; **38**: 1321–1324.
21 Jin JJ, Nakura J, Wu Z, Yamamoto M, Abe M, Tabara Y *et al.* Association of endothelin-1 gene variant with hypertension. *Hypertension* 2003; **41**: 163–167.
22 Tatchum-Talom R, Martel C, Labrie C, Labrie F, Marette A. Gender differences in hemodynamic responses to endothelin-1. *J Cardiovasc Pharmacol* 2000; **36**(suppl 1): S102–S104.
23 Jiang C, Sarrel PM, Poole-Wilson PA, Collins P. Acute effect of 17-estradiol on rabbit coronary artery contractile responses to endothelin-1. *Am J Physiol* 1992; **263**: H271–H275.
24 Lajemi M, Gautier S, Poirier O, Baguet JP, Mimran A, Gosse P *et al.* Endothelin gene variants and aortic and

cardiac structure in never-treated hypertensives. *Am J Hypertens* 2001; **14**: 755–760.

25 Funalot B, Courbon D, Brousseau T, Poirier O, Berr C, Cambien F *et al.* Genes encoding endothelin-converting enzyme-1 and endothelin- interact to influence blood pressure in women: The EVA study. *J Hypertens* 2004; **22**: 739–743.

26 Blacher J, Protogerou AD, Safer ME. Large artery stiffness and antihypertensive agents. *Curr Pharm Des* 2005; **25**: 3317–3326.

27 Campia U, Cardillo C, Panza JA. Ethnic differences in the vasoconstrictor activity of endogenous endothelin-1 in hypertensive patients. *Circulation* 2004; **109**: 3191–3195.

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Successful Treatment of Primitive Neuroectodermal Tumor-associated Microangiopathy with Multiple Bone Metastases

Toru Morishita[1], Masanori Matsumoto[2], Kanya Honoki[3], Atsushi Yoshida[3], Yoshinori Takakura[3] and Yoshihiro Fujimura[2]

[1]Department of Orthopedic Surgery, National Hospital Organization Nara Medical Center, Nara, [2]Department of Blood Transfusion Medicine and [3]Department of Orthopedic Surgery, Nara Medical University, Nara, Japan

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We report here a 16-year-old male with primitive neuroectodermal tumor (PNET)-associated probable microangiopathy with multiple bone metastases. Laboratory findings excluded the possibility of amegakaryocytic or immune thrombocytopenia and/or disseminated intravascular coagulation. He was first treated with plasma-exchange (PE), followed by platelet transfusions, steroid pulse therapy and combined chemotherapy. PE and steroid pulse therapy reduced his plasma CRP level. Combined chemotherapy drastically increased his platelet count until it had almost normalized without further transfusion. The plasma level of von Willebrand factor-cleaving protease (ADAMTS13) activity measured before PE was not severely deficient (48% of normal) and an unusually large von Willebrand factor multimer (UL-VWFM) was detected. We consider that this therapeutic strategy has the following benefits: (1) reduction of plasma levels of factors that are harmful to both platelet activation and endothelial cell injury; and (2) the safe transfusion of platelet concentrate in thrombotic microangiopathy. This strategy should be confirmed in further cases.

Key words: PNET – microangiopathy – chemotherapy – ADAMTS13 – UL-VWFM

INTRODUCTION

Malignancy-associated thrombotic microangiopathy (TMA), characterized by thrombocytopenia and microangiopathic hemolytic anemia, is a rare but life-threatening complication of sarcoma and its treatment remains controversial. Recent studies, however, have indicated that such patients usually have normal plasma von Willebrand factor-cleaving protease (ADAMTS13) activity (1,2), and that platelet transfusions are generally contra-indicated in these patients because transfusions have been associated with disease exacerbation (3,4). We report here a case of PNET-associated probable TMA that was successfully treated by platelet transfusion after extensive plasmapheresis (PE) followed by chemotherapy.

CASE REPORT

The patient was a 16-year-old male (body weight, 60 kg) who had complained of a high fever and fatigue beginning in June 2002. He was admitted to a nearby hospital on 14 June and received penicillin injections for three days. Suspicion of meningitis, sepsis, viral infection and immunologic diseases was excluded by negative results of leucocytosis in the cerebrospinal fluid, culture of blood and cerebrospinal fluid and antibodies against certain viruses and nucleus. A lytic area in the right eighth rib was then noted on radiography. Bone scintigraphy showed multiple hot lesions on 18 June. Bone marrow examination performed on 19 June was normal without invasion of malignant cells. Pathological examination using biopsy specimens together with the demonstration of EWS-FLI1 translocation confirmed a diagnosis of a PNET (Fig. 1). On 25 June, he developed slight bilirubinemia (1.2 mg/dl) and thrombocytopenia (94×10^9/l), which then rapidly progressed together with hemolytic signs consisting of rouleaux formation and poikilocytosis of erythrocytes in the peripheral blood, and microscopic hematuria. Normoblasts and immature myeloid cells in the peripheral blood were also found as leucoerythroblastic features. Because of this complex clinical picture, he was transferred to our hospital on 8 July. On admission, he had anemia (Hb 105 g/l) (normal range: 135–176), thrombocytopenia (26×10^9/l), and high serum levels of CRP

For reprints and all correspondence: Toru Morishita, Department of Orthopedic Surgery, National Hospital Organization Nara Medical Center, 2-789 Shichijo, Nara, Nara 630-8053; Japan. E-mail: morishit@wnara.hosp.go.jp

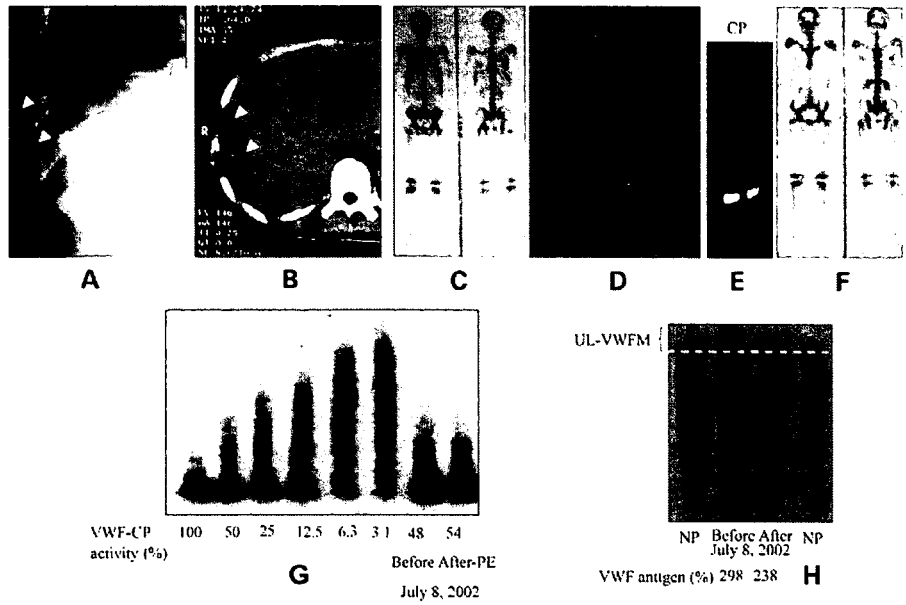

Figure 1. Results of clinical and diagnostic images. Plain radiograph (A) and CT (B) showing a lytic lesion and large expansive tumor in the right eighth rib. Bone scintigram (C) showing multiple areas of abnormal uptake in the skull, spine, pelvis, ribs, shoulders and knees. Histology of the biopsy specimen (D) was compatible with primitive neuroectodermal tumor. Demonstration of EWS-FLI1 translocation (E) by reverse transcription of polymerase chain reaction; c, positive control; p, patient. Bone scintigram, 10 months after diagnosis (F), did not show any abnormal uptake. Assay of plasma VWF-cp activity before and after plasma exchange (PE) on 8 July 2002 (G). Detection of unusually large von Willebrand factor multimers (UL-VWF) and VWF antigen level in the patient plasma before and after PE on 8 July 2002 (H). Note that the plasma VWF antigen level decreased after PE.

(251 mg/l), LDH (1787 U/l) (normal range: 106–211 U/l), GOT (49 U/l) (normal range: 12–32 U/L), and ALP (2,100 U/l) (normal range: 200–760 U/l). Other laboratory findings were as follows: BUN 6.78 μmol/l (normal range: 2.85–7.12 μmol/l), creatinine 53.04 μmol/l (normal range: 26.52–79.56 μmol/l), and total bilirubin 18.8 μmol/l (normal range: 5.1–18.8 μmol/l). His blood type was O-Rho (D) positive and both direct and indirect Coombs tests were negative. Antiplatelet antibody determined by mixed passive hemaglutination assay was negative. He had never previously undergone chemotherapy or blood transfusion. Coagulation screening tests including the levels of antithrombin (86%) and fibrinogen (5.91 g/l) were within normal ranges; however, his serum FDP level had increased slightly to 43.7 μg/ml. Plasma ADAMTS13 activity determined by the multimer assay was not immediately available. Based on these clinical and laboratory findings, we suspected that the patient had PNET-associated TMA rather than immune thrombocytopenia or disseminated intravascular coagulation (DIC). Because of his extremely poor general condition, surgical and/or chemotherapeutic approaches were thought to be inadvisable.

Thus, we prepared a protocol consisting an initial plasma exchange (PE) followed by transfusion of a single-donor platelet concentrate (PC) supplied by the Japan Red Cross Blood Center. This regimen was repeated for five consecutive days, together with steroid pulse therapy (methyl prednisolone 1 g/d for 3 days). Using this approach, PC was transfused without any appreciable adverse reactions. The expected rise in platelet count was identified 1 h after each infusion. It then decreased to the pre-infusion level ($23–33 \times 10^9$/l) over the next few days. Bone marrow examination on the fourth hospital day (11 July) demonstrated a normal nuclear cell count (137×10^9/l), of which malignant cells accounted for 29.5%. Meanwhile, the megakaryocyte count was normal or had increased slightly (200/μl), supporting the concept of enhanced consumption of newly-produced platelets. After sequential PE, a marked decrease in the CRP level was observed, and the general condition of the patient appeared to improve. However, the LDH level remained elevated and even increased slightly while the anemia worsened, indicating invasive expansion of tumor cells. Thus, on 13 July, we started combined chemotherapy, consisting of vincristine (VCR), adriamycin (ADR), and cyclophosphamide (CPA), that resulted in a dramatic increase in the platelet count with a concomitant decrease in LDH. Furthermore, the anemia ceased to progress, with no red blood cell transfusion required throughout this clinical course. Partial response was confirmed by resection of the right eighth rib after chemotherapy. Total body and local irradiation was performed after the Hi-MEC regimen, resulting in an absence of abnormal accumulation on bone scintigraphy 10 months after diagnosis. However, 13 months after diagnosis, recurrences in the right hip joint and orbit were detected and the patient died of disease. Survival after diagnosis was 23 months.

Before PE, his plasma VWF antigen level was elevated (298%) and an unusually-large VWF multimer (UL-VWFM) was present (Fig. 2).

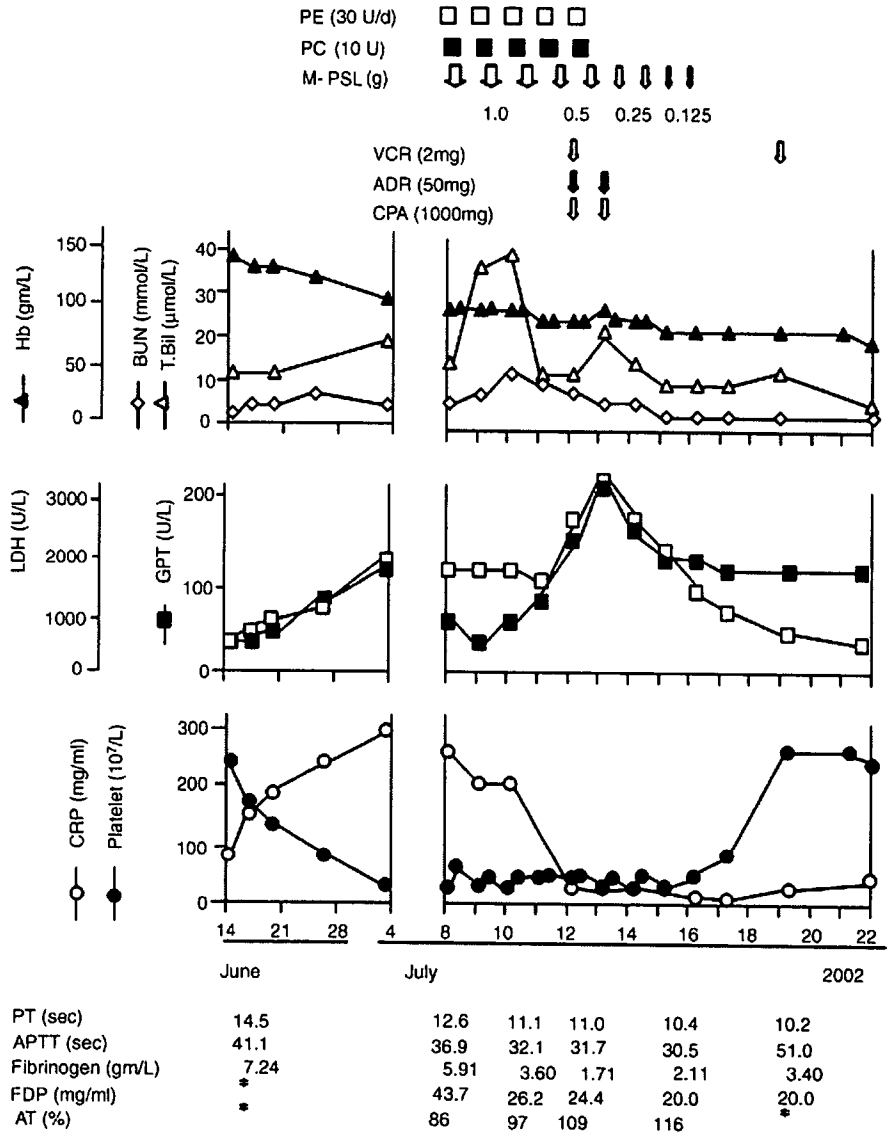

Figure 2. Time course of laboratory parameters and therapeutic regimen. PE, plasma exchange; PC, platelet concentration; m-psl, methyl prednisolone; VCR, vincristine; ADR, adriamycin; CPA, cyclophosphamide; Hb, hemoglobin; BUN, blood urea nitrogen; T.Bil, total bilirubin; LDH, lactate dehydrogenase; GPT, glutamic-pyruvic transaminase; CRP, C-reactive protein; PT, prothrombin time; APTT, activated partial thromboplastin time, FDP; fibrinogen degradation products; AT, antithrombin.

DISCUSSION

The diagnosis of TMA was based on schistocytosis and evidence of hemolysis. In our case, an elevated level of LDH was evident; however, schistocytosis was not tested in our hospital.

Thrombocytopenia occurred as a result of low platelet production and/or increased breakdown. In our case, a normal or slight increase in the production of platelets in the bone marrow was confirmed during hospitalization. Therefore, the increased breakdown of platelets was assumed. Spherocytes in the peripheral blood, which are characteristic in immune and hereditary hemolysis, were not found before transition. Considering these data along with the negative results on direct and indirect Coombs tests, the possibility of immune hemolysis was considered highly improbable and DIC was also excluded by the absence of signs indicating decreased ATIII.

Though a high CRP value persisted, severe infection, including meningitis, sepsis and viral infection were excluded by intensive examination. High fever and leucocytosis were thus considered characteristic symptoms of PNET and not owing to infection. Splenomegaly as a sign of increased breakdown of platelets was not confirmed by CT. There was no history of blood transfusion.

Invasion of malignant cells as confirmed by bone marrow examination occurred between 11 June and 19 July, and thrombocytopenia with hemolytic anemia occurred concomitant with this invasion, though multiple bone metastases had

already been confirmed by bone scintigraphy. Therefore the formation of bone metastases is insufficient to explain thrombocytopenia in this case. We consider that thrombocytopenia was probably as a result of malignant tumor-associated TMA.

Detection of UL-VWFM, released from endothelial cells and cleaved by ADAMTS13, and subnormal activity of ADAMTS13, reported as a marker to differentiate between TTP and HUS (3), were also confirmed later. Detection of UL-VWFM suggests injury of the endothelial cells or obstruction of cleavage by ADAMTS13. In our case, ADAMTS13 activity was subnormal, which agreed with the findings in the majority of TMA reported (1). Therefore, detection of UL-VWFM suggested injury of endothelial cells (2). Invasion of malignant cells, synchronously occurring, may have caused endothelial cell injury. Histological examination may help clarify the mechanism.

Cytokines have recently been reported to mediate UL-VWFM release from vascular endothelial cells (5). Furthermore, it was proposed that cytokines that injure vascular endothelial cells may interfere with the efficient supply of ADAMTS13 (5). Thus, cytokines may be another cause of TMA.

Systemic chemotherapy is usually indicated except in cases of chemotherapy-induced TMA. However, low platelet count made the initiation of this therapy inadvisable. Thus, transfusion of platelets was performed after PE to prevent adverse reaction. It has been proposed that PC transfusion is contra-indicated in TMA because uncleaved UL-VWFM induces platelet aggregation under high shear stress and exacerbates thrombosis (3). However, after removal of factors including UL-VWFM and cytokines from the circulation by PE, PC transfusion was performed safely and quickly resulted in raising the platelet count over the short time. However, the basic conditions, for example, expansion of tumor cells, may gradually lower the platelet count again. Thus, treatment of the tumor itself is necessary. Combined chemotherapy after PE dramatically improved TMA in our case. This also supports the hypothesis that malignant cells were related to injury of the endothelial cells. Prognosis of TMA depends on the chemosensitivity of the tumor itself. Further experience is necessary to confirm this regimen.

The present findings may improve our understanding the reason why malignancy-associated TMA responds poorly to PE therapy alone, as has been commonly accepted.

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Conflict of interest statement

None declared.

References

1. Fontana S, Gerritsen E, Hovinga KJ, Furlan M, Lammle B. Microangiopathic hemolytic anemia in metastatizing malignant tumor is not associated with a severe deficiency of the von Willebrand factor-cleaving protease. *Br J Haematol* 2001;113:100–2.
2. Blot E, Decaudin D, Veyardier A, Bardier A, Zagame OL, Pouillart P. Cancer-related thrombotic microangiopathy secondary to Von Willebrand factor-cleaving protease deficiency. *Thromb Res* 2002;106:127–30.
3. Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, et al. Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 1998;339:1578–84.
4. Allford SL, Hunt BJ, Rose P, Machin SJ. Guidelines on the diagnosis and management of the thrombotic microangiopathic haemolytic anaemia. *Br J Hematol* 2003;120:556–73.
5. Bernardo A, Ball C, Nolasco L, Moake JF, Dong J-F. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood* 2004;104:100–6.

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REGULAR ARTICLE

Decreased ADAMTS13 activity in plasma from patients with thrombotic thrombocytopenic purpura

Toshihiko Kobayashi [a], Hideo Wada [b,*], Yuko Kamikura [a], Takeshi Matsumoto [a], Yoshitaka Mori [c], Toshihiro Kaneko [a], Tsutomu Nobori [b], Masanori Matsumoto [d], Yoshihiro Fujimura [d], Hiroshi Shiku [a]

[a] *Second Departments of Internal Medicine, Mie University Graduate School of Medicine, Tsu, Japan*
[b] *Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan*
[c] *Mie Red Cross Blood Center, Tsu, Mie, Japan*
[d] *Department of Blood Transfusion Medicine and Department of Health Science, Nara Medical University, Kashihara, Nara, Japan*

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KEYWORDS
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TTP

Abstract The ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13) activity was measured by a fluorescence resonance energy transfer (FRET) assay in the plasma of healthy volunteers and thrombotic thrombocytopenic purpura (TTP) patients to examine its usefulness in the diagnosis of TTP.

The plasma levels of the ADAMTS13 activity did not show a normal distribution. Its median value was 107% (range: 55–170%) in healthy volunteers, but was significantly lower in patients with TTP (acquired or familial) and in patients with hematopoietic stem cell transplantation. However, it was not significantly lower in patients with antiphospholipid syndrome (APS). The ADAMTS13 activity by a FRET assay was closely correlated with that by the ADAMTS13 multimer method ($r=0.816$; $p<0.001$). In 18 patients with less than 10% of ADAMTS13 activity by FRET assay, less than 10% of that by multimer assay was 16, thus suggesting a good correlation for a low level of ADAMTS13.

These findings suggest that the ADAMTS13 FRET assay correlates well with the ADAMTS13 multimer method and it is therefore useful for making a diagnosis of TTP.

* Corresponding author. Tel.: +81 59 232 1111; fax: +81 59 231 5204.
E-mail address: wadahide@clin.medic.mie-u.ac.jp (H. Wada).

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening syndrome characterized by thrombocytopenia and microangiopathic hemolytic anemia, and is often associated with neurological dysfunction, renal failure, and fever [1,2]. Unusually large von Willebrand factor (VWF) multimers produced in and then quickly released from vascular endothelial cells, have often been found in patients plasma in familial and nonfamilial TTP [3,4]. VWF is a large glycoprotein which is essential for high-shear stress associated platelet adhesion and aggregation [5]. These large VWF multimers have been thought to interact with circulating platelets, thus resulting in platelet clumping due to an elevated shear stress [3]. Furlan et al. [6] and Tsai [7] independently showed the plasma vWF to be physiologically cleaved by specific metalloprotease. Thereafter, metalloprotease was purified, and cDNA sequencing identified the enzyme as ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13) is a metalloprotease, that specifically cleaves the multimeric VWF [8–12].

A severely deficient ADAMTS13 activity (less than 5% of that in normal plasma) is caused by either a mutation of the ADAMTS13 gene [9,13] or by inhibitory antibodies against ADAMTS13 [14–16]. Although measuring the ADAMTS13 activity is important in the TTP diagnosis, the existing methods require time and skill. Kokame et al. [17] developed a synthetic 73-amino-acid peptide, the FRETS-VWF73, and cleavage of this substrate between two modified residues relieves the fluorescence quenching in the intact peptide. Kokame developed a fluorescence resonance energy transfer (FRET) assay for ADAMTS13 activity [17]. This assay is very easy to perform and it is not time consuming, thus suggesting that it is useful for clinical application.

In this study, we measured the ADAMTS13 activity by a FRET assay in the plasma of healthy volunteers and TTP patients and thus examined the usefulness of a diagnosis of TTP.

Materials and methods

The ADAMTS13 activity was measured in 68 healthy volunteers (19 females and 49 males; median age, 33 years; range, 20–54 years). The ADAMTS13 activity was also measured in 38 patients with TTP (21 females and 17 males; median age 46 years; range 1–84 years), in 8 patients from a congenital TTP family (5 females and 3 males; median age 54 years; range 24–78 years), in 24 patients with antiphospholipid antibody syndrome (APS) (19 females and 5 males; median age 52 years; range 25–69 years), and in 29 patients after hematopoietic stem cell transplantation (HSCT) (13 females and 16 males; median age 36 years; range 17–52 years).

The diagnosis of TTP was made on thrombocytopenia due to the consumption, microangiopathic hemolytic anemia, neurological abnormalities, renal function impairment and high fever [16]. APS was diagnosed based on the Sapporo criteria [18]. The study protocol was approved by the Human Ethics Review Committee of Mie University School of Medicine and a signed consent form was obtained from each subject.

Whole blood was collected in tubes containing 1/10 volume of 3.8% sodium citrate as an anticoagulant. Plasma was obtained by centrifugation at 3000×*g* at 4 °C for 15 min.

Fluorescent assay to measure the ADAMTS13 activity

The fluorogenic substrate, FRETS-VWF73, was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) [17]. It was dissolved in 25% dimethlsulfoxide/water to prepare the 100 μmol/l stock solution. The assay was performed according to the method of Kokame et al. [17].

Briefly, pooled human plasma (a range of 0–8 μl as a standard) or 4 μl of each test plasma were diluted in 100 μl of assay buffer (5 mmol/l Bis–Tris, 25 mmol/l $CaCl_2$, 0.005% Tween-20, pH 6.0) in the well of a 96-well white plate (Thermo Elecron Corporation; Waltham, USA). Next, 100 μl of 4 μmol/l FRETS-VWF73 in the assay buffer was added to each well. Fluorescence was measured at 30 °C in a Fluoroskan Ascent FL (Thermo Elecron Corporation; Waltham, USA) equipped with a 340 nm excitation filter and a 450 nm emission filter. Fluorescence was measured every 5 min. The

Table 1 Subjects

	Number	Sex (f:m)	Age (range)
Healthy volunteers	68	19:49	36 (20–54)
TTP	38	21:17	46 (1–84)
Acquired	32	18:14	46 (16–84)
Familial (3 families)	6	3:3	26 (1–55)
TTP family (3 families)	8	5:3	54 (24–78)
Antiphospholipid syndrome	24	19:5	52 (25–69)
Hematopoietic stem cell transplantation	29	13:16	36 (17–52)

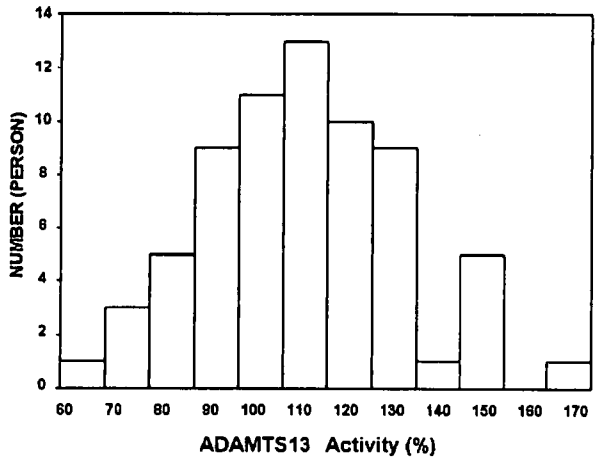

Figure 1 ADAMTS13 activity in healthy volunteers.

reaction rate was calculated by a linear regression analysis of fluorescence over time from 0 to 60 min using a Prism software package (GraphPad Software, San Diego, USA).

The ADAMTS13 activity was determined by the multimetric method which was performed according to the method of Furlan et al. [19,20].

Statistical analysis

The data were expressed as the mean ± standard deviation (S.D.). The differences between the groups were examined for statistical significance using the Mann–Whitney's *U*-test while the correlation between the two variables was tested by Pearson's correlation analysis. A *P* value of less than 0.05 denoted the presence of a statistically significant difference (Table 1).

Results

In healthy volunteers, the plasma levels of the ADAMTS13 activity by FRETS-VWF did not show a

Table 2 TTP patients

No.	Sex	Age	TTP/HUS	FRET	Multimer	Inhibitor	Outcome
1	F	45	TTP	3% >	3% >	(+)	Survive
2	F	17	TTP	3% >	3% >	(+)	Survive
3	F	34	TTP	3% >	3% >	(+)	Survive
4	F	16	TTP	3% >	3% >	(+)	Survive
5	M	38	TTP	3% >	3% >	(+)	Survive
6	M	75	TTP	3% >	3% >	(+)	Survive
7	F	64	TTP	3% >	3% >	(+)	Survive
8	F	17	TTP	3% >	3% >	(+)	Survive
9	F	46	TTP	3% >	3% >	(+)	Survive
10	M	41	TTP	3% >	3% >	(+)	Survive
11	F	45	TTP due to collagen D	3% >	3% >	(+)	Survive
12	F	34	TTP	3% >	3% >	(+)	Survive
13	F	43	TTP	3% >	3% >	(+)	Survive
14	M	55	TTP	3% >	6%	(+)	Survive
15	M	59	TTP	3% >	12%	(+)	Survive
16	M	72	TTP	3% >	3% >	(+)	Death
17	M	79	TTP	3% >	4%	ND	Survive
18	F	50	TTP	14%	28%	ND	Survive
19	M	67	TTP after transplantation	13%	26%	(−)	Death
20	F	71	TTP	28%	60%	(−)	Death
21	F	51	TTP	39%	24%	(−)	Death
22	F	72	TTP	34%	25%	(−)	Death
23	M	17	TTP	49%	70%	(−)	Death
24	F	68	TTP	48%	48%	(−)	Survive
25	F	68	TTP	48	28	(−)	Survive
26	M	48	TTP	58%	28%	(−)	Death
27	M	28	TTP due to collagen D	79	84	(−)	Death
28	F	84	TTP due to drug	80%	52%	(−)	Survive
29	M	69	TTP	97%	70%	(−)	Survive
30	M	44	TTP	100%	26%	ND	Death
31	F	17	TTP with relapse	144%	ND	(−)	Survive
32	F	49	TTP	144%	100%	(−)	Survive
33	M	51	Familial TTP (Family A)	3%>	3%>	(−)	Death
34	F	24	Familial TTP (Family B)	83%	26%	(−)	Survive
35	M	26	Familial TTP (Family B)	113%	42%	(−)	Survive
36	M	1	Familial TTP (Family B)	119%	44%	(−)	Survive
37	F	55	Familial TTP (Family C)	110%	118%	(−)	Survive
38	F	30	Familial TTP (Family C)	120%	150%	(−)	Survive

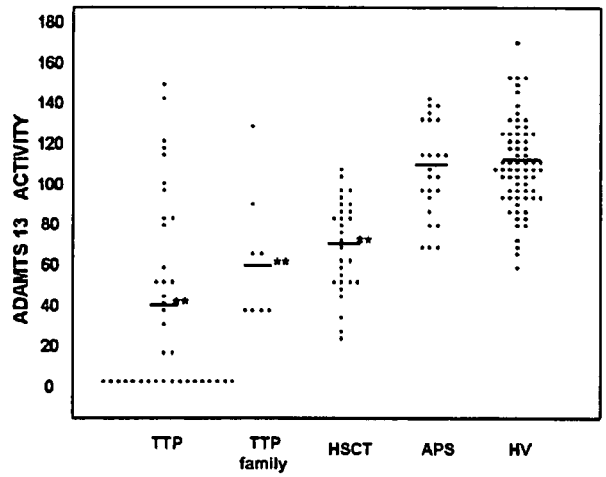

Figure 2 ADAMTS13 activities in TTP, TTP family, HSCT, APS and HV HSCT; hematopoietic stem cell transplantation, APS; antiphospholipid syndrome, HV; healthy volunteer. $^{**}p<0.01$.

normal distribution, and its median value was 106.6% (minimum–maximum; 55–170%) (Fig. 1). In 38 patients with TTP, 6 had familial TTP while 32 had acquired TTP. Ten patients with TTP died within 3 months, while 28 patients had a complete remission. The ADAMTS13 activity of 18 patients (47%) with TTP was less than 5% by FRET assay and the inhibitor for ADAMTS13 was measured in 17 of these patients. 16 patients had an inhibitor for ADAMTS13 and one had familial TTP (Table 2). The ADAMTS13 activity was significantly lower in the patients with TTP (median 13.1%; interquartile range 0–78.5%, $p<0.01$), TTP family (49.4%; 36.2–75.0%, $p<0.01$) and patients with hematopoietic stem cell transplantation (70.0%; 48.4–86.6%, $p<0.01$) than in healthy volunteers (106.7%; 93.7–123.7%). There was no significant difference in the ADAMTS13 activity between patients with APS (70.0%; 48.4–86.6%) and healthy

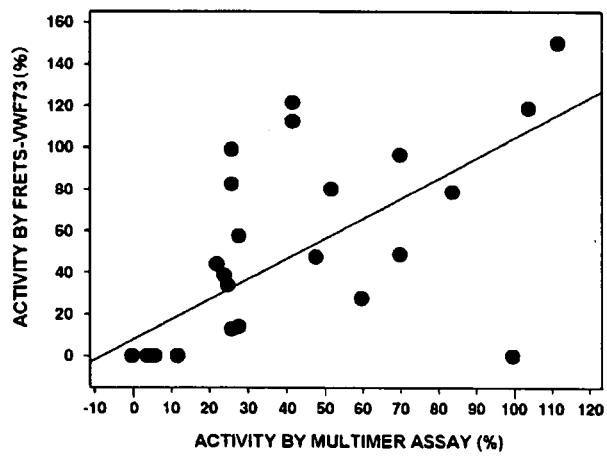

Figure 3 Correlation of ADAMTS13 activity between the FRET assay and multimer assay.

Table 3 Comparison of the ADAMTS13 activity between by the findings of the FRETS-VWF73 and multimer method

		Multimer method		
		0–20%	20–50%	50%<
FRET method	0–20%	18(1)	2	0
	20–50%	0	4	2
	>50%	0	5(3)	6(2)

( ) shows familial TTP.
The ADAMTS13 level by a multimer assay in one case was not measured.

volunteers (Fig. 2). There were 3 families with TTP. The mutation of the ADAMTS13 gene was detected in family A [21] but was not found in the other 2 families. The ADAMTS13 activity by FRETS-VWF73 was more than 50% but according to a multimer assay, it was less than 50% in the family B members.

The ADAMTS13 activity in the members of family C was more than 50%. Marked reduction (less than 3%) of the ADAMTS13 activity was not observed in TTP family, HSCT and APS. The ADAMTS13 activity by FRETS-VWF73 was closely correlated with that determined by the multimer method ($Y=7.913823+0.967015X$, $r=0.816$; $p<0.001$) (Fig. 3). Twenty TTP patients showed less than 20% of ADAMTS13 activity by FRET assay, while 18 showed less than 20% of that by a multimer assay and 2 patients showed 20–50%. In more than 50% of the ADAMTS13 activity by FRET assay, 5 patients showed 20–50% of that by a multimer assay, while 6 showed more than 50% (Table 3).

Discussion

ADAMTS13 was recently identified to be a new hemostatic factor, previously called VWF cleaving protease. Neither the congenital or acquired defects of the enzymatic activity lead to thrombotic thrombocytopenic purpura (TTP). ADAMTS13 specifically cleaves a peptidyl bond between Y1605 and M1606 in the A2 domain of VWF which helps to determine the minimal region which was recognized as a specific substrate by ADAMTS13 [22].

In healthy volunteers, normal range of plasma ADAMTS13 activity by FRETS-VWF ranged from 55% to 170%. The plasma levels of ADAMTS13 did not show a normal distribution, probably because the ADAMTS13 levels were affected by the production in the liver or consumption [3]. The VWF levels decreased in persons with blood type "O" [23].

The ADAMTS13 activity was significantly lower in the patients with TTP and the TTP family, thus indicating that ADAMTS13 plays an important role in the onset of TTP. However, 6 patients had an

ADAMTS13 activity of more than 60%, thus suggesting that the TTP in these patients may have been caused by abnormalities of other factors such as Factor H [24] and CD46 [25]. The activity of ADAMTS13 was low in patients with hematopoietic stem cell transplantation. A decreased activity was reported in patients with hepatic veno-occlusive disease (VOD) after stem cell transplantation [26]. These findings suggest that a reduced amount ADAMTS13 may be a risk factor for the onset of VOD.

The ADAMTS13 activity determined by FRETS-VWF73 was closely correlated with that determined by the multimer method. Especially, in less than 10% of ADAMTS13 activity, these two assays closely correlate. As almost all patients with acquired TTP and showing less than 10% of ADAMTS13 activity had an inhibitor, this FRETS-VWF73 assay may thus be especially useful for TTP patients with an inhibitor. However, there are several discrepancies between the FRET assay and a multimer assay. In 5 cases (3 cases were familial TTP; family B), the ADAMTS13 activity by FRET assay was within the normal range but based on a multimer assay, it was low. This is because a FRET assay can detect the cleaving activity only between Y1605 and M1606 in the A2 domain of VWF, while a multimer assay can detect the cleaving activity of whole VWF. These findings suggest that a FRET assay may miss a few patients with TTP, while a FRET assay may be more sensitive than a multimer assay in some patients.

In TTP patients without an inhibitor, the difference between the two assays may provide important information for a further analysis of ADAMTS13. In addition, an analysis of ADAMTS13 including antigen will thus play an important role in examining various thrombotic diseases.

Acknowledgments

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References

[1] Bukowski RM. Thrombotic thrombocytopenic purpura: a review. *Rev Prog Hemost Thromb* 1982;6:287-337.

[2] Amorosi EL, Ultman JE. Thrombotic thrombocytopenic purpura: report of the 16 cases and review of the literature. *Medicine* 1966;45:139-59.

[3] Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, et al. Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med* 1982; 307:1432-5.

[4] Chow TW, Turner NA, Chintagumpala M, McPherson PD, Nolasco LH, Rice L, et al. Increased von Willebrand factor binding to platelets in single episode and recurrent types of thrombotic thrombocytopenic purpura. *Am J Hematol* 1998;57:293-302.

[5] Ruggeri ZM. Structure and function of von Willebrand factor. *Thromb Haemost* 1999;82:576-84.

[6] Furlan M, Robles R, Lamie B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood* 1996;87:4223-34.

[7] Tsai H-M. Physiologic cleavage of von Willebrand factor by a plasma protease is depend on its conformation and requires calcium ion. *Blood* 1996;87:4235-44.

[8] Soejima K, Mimura N, Hirashima M, Maeda H, Hamamoto T, Nakagaki T, et al. A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? *J Biochem* 2001;130:475-80.

[9] Levy GG, Nichols WC, Lian EC, Foroud T, McClintick JN, McGee BM, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. *Nature* 2001;413:488-94.

[10] Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *J Biol Chem* 2001; 276:41059-63.

[11] Fujikawa K, Suzuki H, McMullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood* 2001;98:1662-6.

[12] Gerritsen HE, Robles R, Lammle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood* 2001;98:1654-61.

[13] Kokame K, Matsumoto M, Soejima K, Yagi H, Ishizashi H, Funato M, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. *Proc Natl Acad Sci U S A* 2002;99:11902-7.

[14] Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, et al. von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 1998;339:1578-84.

[15] Tsai HM, Lian EC. Antibodies to von Willebrand factor-cleaving protease in acute thrombotic thrombocytopenic purpura. *N Engl J Med* 1998;339:1585-94.

[16] Mori Y, Wada H, Gabazza EC, Minami N, Nobori T, Shiku H, et al. Defective von Willebrand factor-cleaving activity on admission is a marker of excellent clinical response to plasma exchange in patients with thrombotic thrombocytopenic purpura. *Transfusion* 2002;42:572-80.

[17] Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. *Br J Haematol* 2005;129:93-100.

[18] Ghirardello A, Doria A, Ruffatti A, Rigoli AM, Vesco P, Calligaro A, et al. Antiphospholipid antibodies (aPL) in systemic lupus erythematosus. Are they specific tool for the diagnosis of aPL syndrome? *Ann Rheum Dis* 1994;53:140-2.

[19] Furlan M, Robles R, Solenthaler M, Wassmer M, Sandoz P, Lammle B. Deficient activity of von Willebrand factor-cleaving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood* 1997;89:3097-103.

[20] Matsumoto M, Yagi H, Ishizashi H, Wada H, Fujimura Y. The Japanese experience with thrombotic thrombocytopenic

purpura–hemolytic uremic syndrome. *Semin Hematol* 2004;**41**:68-74.

[21] Uchida T, Wada H, Mizutani M, Iwashita M, Ishihara H, Shibano T, et al. Identification of novel mutations in ADAMTS13 in an adult patient with congenital thrombotic thrombocytopenic purpura. *Blood* 2004;**104**:2081-3.

[22] Kokame K, Matsumoto M, Fujimura Y, Miyata T. VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. *Blood* 2004; **103**:607-12.

[23] Mannucci PM, Capoferri C, Canciani MT. Plasma levels of von Willebrand factor regulate ADAMTS-13, its major cleaving protease. *Br J Haematol* 2004;**126**:213-8.

[24] Rougier N, Kazatchkine MD, Rougier JP, Fremeaux-Bacchi V, Blouin J, Deschenes G, et al. Human complement factor H deficiency associated with hemolytic uremic syndrome. *J Am Soc Nephrol* 1998;**9**:2318-26.

[25] Noris M, Brioschi S, Caprioli J, Todeschini M, Bresin E, Porrati F, et al. International Registry of Recurrent and Familial HUS/TTP: Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet* 2003;**362**:1542-7.

[26] Park YD, Yoshioka A, Kawa K, Ishizashi H, Yagi H, Yamamoto Y, et al. Impaired activity of plasma von Willebrand factor-cleaving protease may predict the occurrence of hepatic veno-occlusive disease after stem cell transplantation. *Bone Marrow Transplant* 2002;**29**:789-94.

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BRIEF COMMUNICATION

Quantitative Western blot analysis of plasma ADAMTS13 antigen in patients with Upshaw-Schulman syndrome

Hiromichi Ishizashi [a,b], Hideo Yagi [b], Masanori Matsumoto [b], Kenji Soejima [c], Tomohiro Nakagaki [c], Yoshihiro Fujimura [b,*]

[a] *Department of Health Science, Nara Medical University, Nara 634-8522, Japan*
[b] *Department of Blood Transfusion Medicine, Nara Medical University, Nara 634-8522, Japan*
[c] *First Research Department, The Chemo-Sero-Therapeutic Research Institute, Kumamoto 869-1298, Japan*

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KEYWORDS
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USS;
TTP

Upshaw-Schulman syndrome (USS) was originally reported as a disease complex, characterized by chronic thrombocytopenia and hemolytic anemia, that was dramatically improved by infusions of fresh frozen plasma (FFP) [1–6]. USS is now known to be a hereditary deficiency in the activity of von Willebrand factor-cleaving protease (VWF-CP), also known as ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13), and lacks ADAMTS13 autoantibodies (inhibitors) [7]. In contrast, acquired deficiency of ADAMTS13 activity caused by inhibitors is defined as thrombotic thrombocytopenic purpura (TTP), a life-threatening generalized disease characterized by Moschcowitz's pentad [8,9]. Thus, USS is alternatively called congenital TTP, and genetic analysis of *ADAMTS13* has revealed that its mutations are present across the entire gene and not in hot spots [7,10–15]. The *ADAMTS13* gene is located on chromosome 9q34 and USS is a recessive disease, so most USS patients are genetically compound heterozygotes or homozygotes. When expressed in mammalian cells, the *ADAMTS13* gene mutants found in USS patients showed deficient ADAMTS13 activity (ADAMTS13:ACT) that was induced by disturbing the synthesis and/or secretion of the protease. However, these results were left unchecked in the patient plasmas. It was recently shown that the normal plasma level of ADAMTS13 antigen (ADAMTS13:AGN) is approximately 1 μg/ml, according to a sandwich enzyme-linked immunosorbent assay (ELISA) using polyclonal or monoclonal antibodies (mAbs) against ADAMTS13. USS patients exhibit severely reduced levels of ADAMTS13:AGN, resulting in reduced levels of ADAMTS13:ACT [16,17]. However, the investigation of the ADAMTS13 molecules in these patients has not yet been perfomed *in vivo*.

* Corresponding author. Department of Blood Transfusion Medicine, Nara Medical University, 840 Shijyo-cho, Kashihara City, Nara 634-8522, Japan. Tel.: +81 744 22 3051x3289; fax: +81 744 29 0771.
E-mail address: yfujimur@naramed-u.ac.jp (Y. Fujimura).

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We therefore analyzed plasma ADAMTS13:AGN in 9 USS patients and their 25 family members, in whom *ADAMTS13* gene mutations were identified by Western blot (WB) using an anti-ADAMTS13 mAb, WH2-11-1. The epitope of this mAb resides on the 4th thrombospondin-1 domain and is reactive by WB under both reducing and non-reducing conditions [18].

Materials and methods

Assays for ADAMTS13:ACT and ADAMTS13 inhibitors

ADAMTS13:ACT and titers of ADAMTS13 inhibitors (ADAMTS13:INH) were assayed by a novel, highly-sensitive ELISA using a murine mAb (N10-146) specifically recognizing Tyr1605 residue of VWF-A2 domain, generated by ADAMTS13 cleavage, and a recombinant GST-VWF73-His polypeptide as a substrate [19,20]. This ELISA had a limit of detection of 0.5% of the normal ADAMTS13:ACT level in normal pooled plasma, and the average plasma level of ADAMTS13:ACT was 99.1±43.0% (mean±2SD). Inhibitor titers were expressed as Bethesda units (BU), where one inhibitor unit is defined as the amount necessary to reduce ADAMTS13:ACT levels to 50% of the normal levels. Titers of >0.1 BU/ml, as measured by the novel ELISA, were considered significant [20].

Patients

Nine patients from 9 different families (Families A–I) with histories of USS were enrolled in our study. For each family, diagnoses were confirmed by identifying the *ADAMTS13* gene mutations responsible for the disease, as previously described [10,14,15,21]. Of the 25 USS relatives we tested, 23 were definite carriers and 2 were normal subjects.

Citrated plasma samples taken from USS patients were frozen in aliquots at −80 °C until use. For controls, normal citrated plasma was obtained from 60 healthy individuals (30 females and 30 males, aged 20–40 years) and kept in aliquots at −80 °C. Pooled normal plasma was used as the control standard for this study. These studies were conducted with the approval of the Nara Medical University ethics committee.

Characterization of the murine anti-ADAMTS13 mAb WH2-11-1

A murine anti-ADAMTS13 mAb, termed WH2-11-1 (IgG1-κ), was produced by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) using recombinant (r) full-lengh ADAMTS13 as the immunogen [18]. Monoclonal IgGs were purified on a Protein A column (Amersham Biosciences, NJ, USA) according to the manufacturer's instructions. WH2-11-1 recognizes an epitope on the 4th thrombospondin-1 domain, and this was verified using C-terminal truncated rADAMTS13. This mAb detected plasma ADAMTS13:AGN as a 170-kD band by WB under non-reducing conditions and a single 190-kD band under reducing conditions. However, this mAb showed no significant inhibition of ADAMTS13:ACT. In some WB analyses under non-reducing conditions, another anti-ADAMTS13 mAb with an epitope on the disintegrin domain, A10, was also used [22].

Analysis of plasma ADAMTS13:AGN

We quantified plasma ADAMTS13:AGN by WB. Two microliters of undiluted or diluted plasma samples per lane were analyzed after treatment with sample buffer containing SDS and β-mercaptoethanol, followed by separation by reducing 5% SDS polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were blotted onto polyvinylidene difluoride (PVDF) microporous membranes (Immobilon-P, Millipore, MA, USA) using cyclohexylaminopropanesulfonic acid (CAPS)-NaOH buffer (pH11) [23]. We probed the blots for ADAMTS13:AGN with WH2-11-1 as the primary mAb, followed by secondary staining with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Kirkegaaed and Perry Lab, Gaithersburg, MO). After incubation with Western Lighting Chemiluminescence Reagent (PerkinElmer Life Sciences, Shelton, CT), the blots were exposed to X-ray film. Densitometric analysis of ADAMTS13:AGN was performed for the 190-kD band using NIH imageJ (developed by the National Institutes of Health, http://rsb. Info.nih.gov/nih-image/).

Results

When diluted normal plasma was analyzed by WB under reducing conditions, WH2-11-1 detected a single 190-kD band of ADAMTS13:AGN, and the detection limit was determined to be 3% of the normal controls (Fig. 1, top). Densitometric analysis of the 190-kD band showed a nearly straight line on a semi-logarithmic graph (data not shown). Using this assay, the normal range of ADAMTS13:AGN in 60 healthy Japanese subjects (30 females and 30 males, aged 20–40 years) was determined to be 101.6±49.4% (mean±2SD).

Next, plasma from 9 USS patients and 25 of their relatives (23 definite carriers and 2 normal subjects), whose *ADAMTS13* gene mutations had been