A large deletion of the *PROS1* gene in a deep vein thrombosis patient with protein S deficiency

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Summary

Inherited deficiency of protein S encoded by the *PROS1* gene constitutes an important risk factor for deep vein thrombosis (DVT). Nevertheless, although more than 200 deleterious genetic variations in *PROS1* have been identified, causative point mutations of *PROS1* gene are not detected in approximately half of protein S-deficient families. The present study investigated whether there may exist a large deletion in *PROS1* that constitutes a genetic risk factor for Japanese DVT patients. A multiplex ligation-dependent probe amplification analysis was employed to identify the deletions in *PROS1* in 163 Japanese patients with DVT. A large gene deletion was identified in one patient who showed 16% protein S activity and did not carry point mutations in *PROS1* by DNA sequencing and it was validated by the quantitative PCR method. The deletion spanned at least the whole *PROS1* gene (107 kb) and at most from the centromere located downstream of *PROS1*, to before the D3S3619 marker, the first heterozygous marker in the upstream of *PROS1* in chromosome 3. In conclusion, a large deletion in *PROS1* was shown to partly account for DVT with protein S deficiency. Screening for large deletions in *PROS1* might be warranted in *PROS1* causative point mutation-negative DVT patients with protein S deficiency.

Keywords

Gene deletion, deep vein thrombosis, protein S deficiency, *PROS1*, MLPA

Thromb Haemost 2007; 98: 783–789

Introduction

Imbalance between procoagulant and anticoagulant potencies triggers coagulation. Protein S (PS) is a cofactor for the anticoagulant protease, activated protein C (PC), which proteolytically inactivates activated coagulation factors V and VIII. Therefore, inherited deficiency of PS constitutes an important risk factor for venous thrombosis (1). In the general Japanese population, PS deficiency is relatively common, and its estimated prevalence, 1.12%, is much higher than that in the Caucasian population (0.16–0.21%) (2–4). Recently, we and others identified a PS K196E mutation (PS Tokushima) as a genetic risk factor for the development of deep vein thrombosis (DVT) (5–8).

The gene for PS (*PROS1*) is located near the centromere on chromosome 3. It spans approximately 101 kb of genomic DNA and comprises 15 exons and 14 introns (9–12). Human DNA contains the active *PROS1* gene at 3q11.2 and a closely linked pseudogene (*PROSP*) at 3p21-cen that shows 96.5% homology to exons 2 to 15 of the *PROS1* gene. To date, more than 200 deleterious mutations in the *PROS1* gene have been reported (13). Most of the gene defects are small point mutations, such as missense, nonsense, frameshift, or splice site mutations, which are

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Financial support:
This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan, a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Tong Yin, M.D., is a recipient of Takeda Foundation, from Institute of Geriatric Cardiology, General Hospital of People's Liberation Army, Beijing, China.

Received March 21, 2007
Accepted after resubmission July 25, 2007

Prepublished online September 10, 2007
doi:10.1160/TH07–03–0211

scattered throughout the coding region of the *PROS1* gene. Some studies have reported that causative point mutations in *PROS1* were found only in approximately 50% of the cases with functional low PS activity (14). One possible cause of such PS deficiency is the existence of large deletions or inversions covering all or part of the *PROS1* gene, which are difficult to identify by current PCR-based sequencing methods. Several studies in PS-deficient families with *PROS1* causative point mutation-negative members indicated that large deletions of *PROS1* have been identified in patients with PS deficiency and screening for large deletions in *PROS1* causative point mutation-negative individuals is therefore warranted (15–18).

A wide range of methods are available for gene dosage measurement to detect large deletions or insertions, but no one method offers overwhelming advantages. Recently developed technique, multiplex ligation-dependent probe amplification (MLPA), based on PCR amplification of ligated probes hybridized to the target DNA (19, 20), would be beneficial for detecting large cryptic genetic variations. This method has proven to be accurate and reliable for identifying deletions, duplications, and amplifications in several diseases (21–23). In this study, we utilized the MLPA method to determine whether there might be large deletions in *PROS1* that constitute genetic risk factors for DVT in Japanese patients.

Materials and methods

Patients

One hundred sixty-three DVT patients, 78 men and 85 women, were registered by the Study Group of Research on Measures for Intractable Diseases, working under the auspices of the Ministry of Health, Labor, and Welfare of Japan. Six centers (one each in Tochigi, Tokyo, Nagoya, and Kyoto, and two in Osaka) participated in this study (5). The protocol for the study was approved by the ethical review committee, and only those subjects who provided written informed consent for genetic analysis were included.

Clinical profile of a patient with a large *PROS1* gene deletion

A previously healthy 22-year-old female was admitted to our hospital due to swelling of her left limb for the past two months. She had no family history of thrombotic diseases. On physical examination, she showed no abnormal findings except swelling of the left thigh and calf.

Laboratory examination showed that the levels of free PS antigen and PS activity had decreased to 2.1 μg/ml (normal range: 6.5–9.8 μg/ml) and 16% (normal range: 65–105%), respectively, while antithrombin (112.7%; normal range: 80–120%), PC (119%; normal range: 75–125%) and plasminogen (104.8%; normal range: 70–120%) were within the normal ranges. ^{99m}Tc-MAA scintigraphy revealed obstruction of the left common iliac and bilateral popliteal veins with no evidence of pulmonary embolisms. Because of the presence of DVT, decreased level of PS, and exclusion of other underlying diseases causing thrombosis, the patient was diagnosed with PS deficiency.

Laboratory values measurement

Some of the patients' plasma samples were stored at −80°C. We measured the PC amidolytic activity (24) and plasma PS activity using Staclot protein S (Diagnostica Stago, Asnieres, France) (3) in 34 samples of DVT patients.

MLPA method

MLPA was performed as described by Schouten et al. (19) using SALSA MLPA KIT P112 *PROS1* (MRC-Holland, Amsterdam, the Netherlands), a kit for screening deletions or duplications in the *PROS1* gene. The kit contains probes for 13 out of 15 exons in *PROS1*, one probe for the promoter located 6.5 kb upstream of *PROS1*, and one probe for exon 4 in *PROSP*. It also contains 17 control probes located on different chromosomes, 1p36, 2q14, 2q24, 3p22, 5q35, 7q31, 7q, 9p21, 10p12, 12p13, 12q14, 14q22, 15q21, 16q22, 18q21, 19p13, and 21q11. The ligation products were amplified by PCR using the common primer set with the 6-FAM label distributed by the supplier. Approximately 100 ng of genomic DNA was utilized for one MLPA reaction. Amplification products were run on an ABI PRISM 3130 DNA Sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems, Foster City, CA, USA) and analyzed by GeneMapper Software 5.0 (Applied Biosystems).

Dosage analyses based on a comparison between deleted and reference wild-type DNA samples were performed on an Excel software Coffalyser V3 (MRC-Holland). Briefly, all *PROS1* peak areas were normalized by dividing each peak area by the combined areas of all the control probe peaks in the same sample. Then, these normalized peak areas were divided by the corresponding wild-type normalized peak areas (average of five independent normal DNA samples) of that probe amplification product, in order to obtain a series of patient-to-normal DNA copy number ratios. The MLPA analysis was repeated three times for all samples.

Q-PCR analysis

DNA copy numbers for 14 exons in *PROS1* and exon 4 in *PROSP* were determined by Q-PCR. Primers for each sequence (except for the sequence of exon 1) were designed to avoid concurrent amplification of the *PROSP* sequence. The amplicon length ranged from 67 to 198 bps. The PCR reaction and the reference genes were as described previously (25). Each assay included a no-template control (in duplex), 20 ng of normal calibrator human genomic DNA (Roche Applied Science, Indianapolis, IN, USA) (in quadruplicate), and 20 ng of sample DNA (in quadruplicate). Calculation of the gene copy number was performed using the comparative (delta-C_t) C_t method (26) and the Q-PCR automated analysis software package "qBase" (http://medgen.ugent.be/qbase/). A haploid copy number of 1 is expected for a normal sample and a value of ≤0.5 for a sample with deletion.

PROS1 sequence analysis

A genomic DNA sample from the DVT patient with the deletion in *PROS1* was sequenced for all exons in *PROS1* using an ABI 3730 sequencer (Applied Biosystems) (27). Genetic variations were identified with NAMIHEI (Mitsui Knowledge Industry Co., Ltd., Tokyo, Japan) and Sequencher (Gene Codes Corpor-

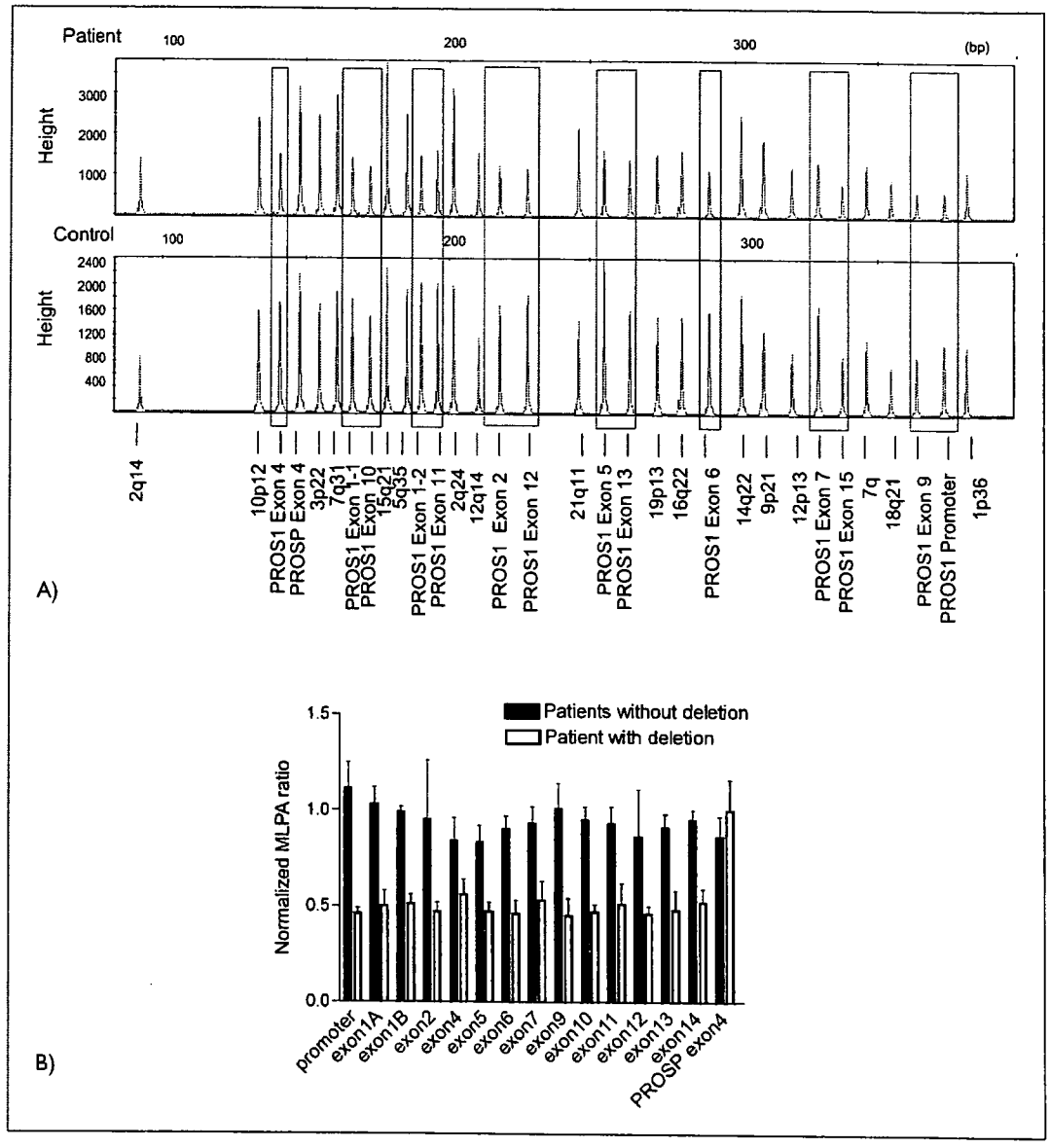

Figure 1: MLPA analysis of the deletion in the *PROS1* gene.
A) The representative MLPA electropherograms in one patient with the deletion in *PROS1* (top) and in a normal control DNA (bottom). Deleted exons are marked by squares. This is one representative of three rounds of MLPA analyses. Probes are 13 out of 15 exons in *PROS1* (two probes for exon 1), one probe for promoter located 6.5 kb upstream of *PROS1*, one probe for exon 4 in *PROSP* and 17 controls located on different chromosomes. B) The representative histograms showing a deletion of 14 probes in *PROS1* in one patient (white bars) and the average normalized MLPA ratio in the patients without deletion (black bars). The probe for exon 4 in *PROSP* is shown as the control probe. The data are presented as the means ± SD from three rounds of MLPA.

ation, Ann Arbor, MI, USA) software, followed by visual inspection (28).

SNPs genotyping and microsatellite analysis

Five SNP markers located within a 252 kb region centered on *PROS1* were chosen on the basis of the available data from NCBI Map Viewer (Build 36.2) and the genotyping of the markers was performed by the TaqMan-PCR genotyping system (29).

Four microsatellite markers (D3S3681, D3S1276, D3S3634 and D3S1271) covering a 22 Mbp region centered on the *PROS1* locus were obtained from the ABI Linkage Mapping Set Version 2.5 (Applied Biosystems), and the microsatellite marker analyses were performed according to the manufacturer's instructions. For the other five microsatellite markers (D3S3556, D3S1251, D3S3619, D3S1752 and D3S3716) in the region, we designed PCR primer sets. Primer sequences were available on request. PCR products were detected by Agilent Bioanalyzer 2100 (Agilent Technologies, Boeblingen, Germany) or by electrophoresis through the 12% polyacrylamide gel stained with SYBR Green I (Nucleic Acid Gel Stain (Molecular Probes, Eugene, OR, USA). The products were also directly sequenced to reveal the allele zygosity.

Results

Deletion detection using MLPA analysis

To identify the large deletions in *PROS1* in patients with DVT, we utilized MLPA analysis. This method has the advantage of using a relatively small amount of DNA – i.e. only 100 ng of genomic DNA are required for one MLPA analysis to reveal a *PROS1* gene deletion. A deletion of one copy of a probe target sequence is usually apparent by a 35–55% reduction in relative peak area. A gain in copy number from two to three copies is usually apparent by a 30–55% increase in relative peak area.

We identified reduced peak areas in the *PROS1* regions in one DVT patient, which indicates a heterozygous deletion encompassing the whole *PROS1* gene (Fig. 1). No significant changes were found in the peak areas of the control probes in-

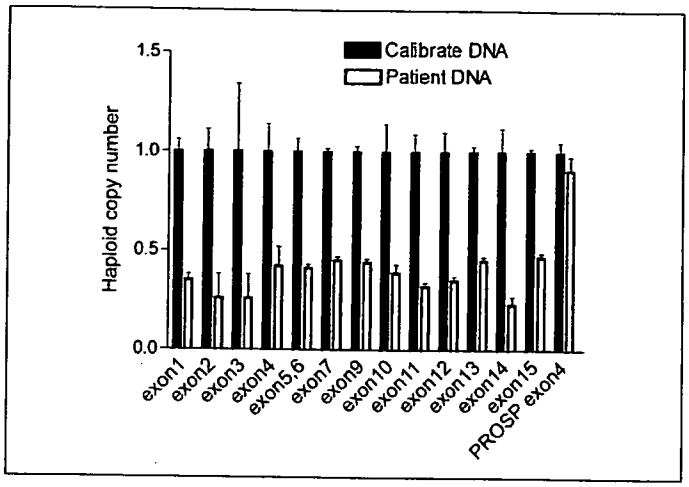

Figure 2: Haploid copy number status of *PROS1* determined by Q-PCR. Representative histogram showing the haploid copy numbers of *PROS1* exons in one patient with the *PROS1* deletion (white bars) and in the normally calibrated DNA (black bars). Data are presented as the means ± SD from Q-PCR reaction in quadruplicate. The copy number of *PROSP* exon 4 is plotted as no deletion control.

cluding *PROSP* exon 4 in this patient. For other patients, no reduced or increased peak areas were detected. The results of the MLPA analysis were reproducible, with three rounds of MLPA yielding identical findings in all patients. Based on the MLPA analysis, a minimum distance of *PROS1* deletion in this patient was estimated to be 107 kb between the *PROS1* promoter and exon 15 MLPA probes.

Deletion detection using Q-PCR

To confirm the deletions detected by MPLA in the patient, the patient's genomic DNA and a normally calibrated DNA were subjected to Q-PCR analysis to determine the copy numbers of *PROS1*. The analysis confirmed that all exons of *PROS1* were deleted in this patient (Fig. 2). No deletion was detected in *PROSP* exon 4.

Direct sequencing of the coding regions of *PROS1*

As described in *Methods*, PS antigen and PS activity of the patient with the deletion in *PROS1* decreased to 2.1 μg/ml (normal range: 6.5–9.8 μg/ml) and 16% (normal range: 65–105%), respectively, while antithrombin, PC and plasminogen were within normal ranges. This suggested that the patient might be a compound heterozygote for PS deficiency. To identify additional genetic variations in *PROS1* in the patient, we sequenced all exons of *PROS1* in this patient, but no point mutations were found.

Distribution of microsatellites and SNP genotypes

To define the range of deletions detected by the MLPA analysis, five SNP markers and nine microsatellite markers covering a 22 Mbp window centered on the *PROS1* locus were analyzed for their allele zygosities (Table 1). When the allele carries a deletion, it will be hemizygous for all markers within the deleted region, but will appear homozygous. Thus, if a specific marker is homozygous, there might be a deletion covering that locus, but if a specific marker is heterozygous, there must be no deletion covering that locus.

The five SNP markers flanking *PROS1* were homozygous. The marker D3S3619, which was located 1,949 kb upstream of *PROS1*, was detected as the first microsatellite heterozygosity in the upstream region of *PROS1* (Table 1). As a result, the boundary of the deletion in this patient must be within the range of D3S3619 to D3S1276. Since the centromere is located between *PROSP* and exon 15 of *PROS1*, one breakpoint of the deletion must be closer to exon 15 than to *PROSP* (Fig. 3). Therefore, the

Marker	Location, kbp	Allele or repeats	Minor allele frequency	Hetero-zygosity, %	Zygosity in the patient
D3S3681	79894	CA	—	—	heterozygous
D3S1276	85338	CA	—	—	heterozygous
rs9289536	95031	C/T	0.11	0.20	homozygous
rs9713061	95074	G/T	0.10	0.15	homozygous
rs6786905	95213	C/A	0.10	0.18	homozygous
rs13100168	95220	C/T	0.06	0.12	homozygous
rs10433405	95283	G/A	0.14	0.24	homozygous
D3S3556	95525.66	CA	—	—	homozygous
D3S3634	95525.74	CA	—	—	homozygous
D3S1251	95760	CA	—	—	homozygous
D3S3619	97124	CA	—	—	heterozygous
D3S1752	99228	GAT	—	—	heterozygous
D3S3716	100219	CA	—	—	heterozygous
D3S1271	102217	CA	—	—	heterozygous

Minor allele frequencies and heterozygosities of SNPs were calculated from 44 unrelated normal Japanese studied in the International HapMap project. Locations are from NCBI Map Viewer build 36.2. The zygosity analysis was performed for a patient with a deletion in *PROS1*.

Table 1: Description of microsatellite and SNP markers.

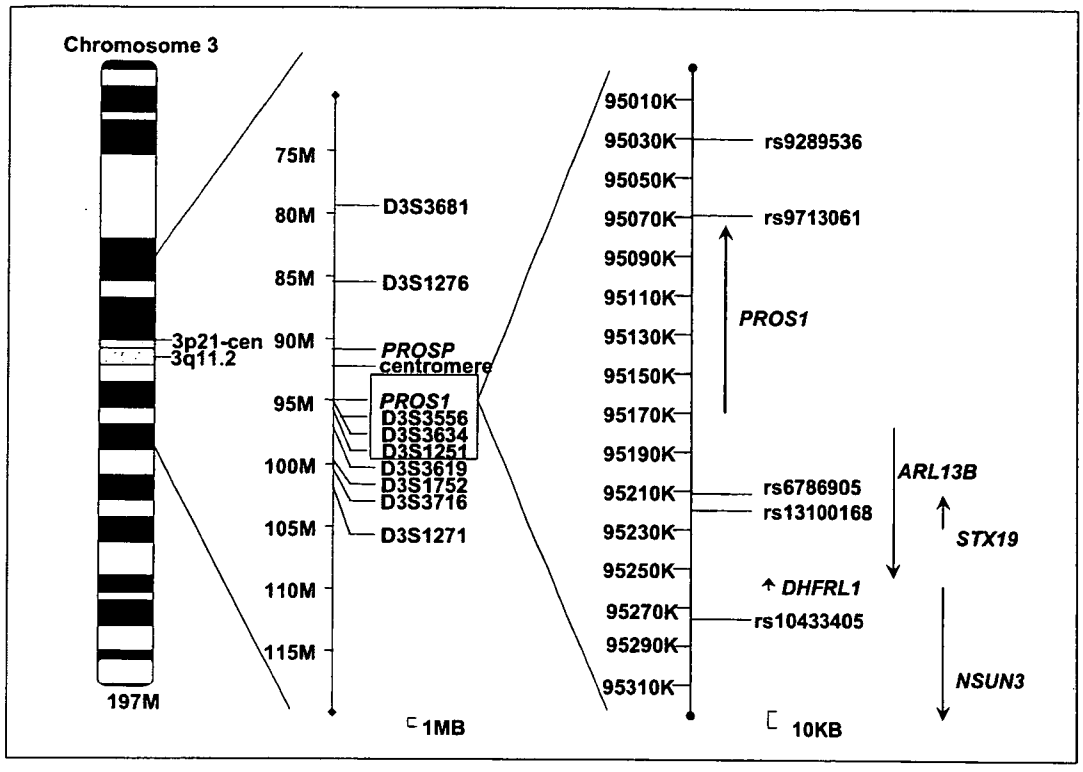

Figure 3: Schematic presentation of the large *PROS1* deletion on chromosome 3. The distributions of *PROS1* (3q11.2), *PROSP* (3p21-cen), *PROS1* flanking SNP and microsatellite markers are mapped on chromosome 3. Possible maximum range of the deletion identified in the patient with DVT is boxed with a grey square. Transcriptional orientations of the genes are shown by arrows. Nucleotide positions are according to NCBI Map Viewer (Build 36.2).

deletion ranged at a maximum from the centromere to before the D3S3619 marker, the first heterozygous marker in the upstream of *PROS1* in chromosome 3. If we take this maximum deletion into account, the large deletion could also include the genes *ARL13B* (ADP-ribosylation factor-like 13B), *STX19* (syntaxin 19), *DHFRL1* (dihydrofolate reductase-like 1), and *NSUN3* (NOL1/NOP2/Sun domain family, member 3), because they are located between *PROS1* and the marker D3S3556 (Fig. 3).

PROS1 deletion carrier in *PROS1* causative point mutation-negative Japanese DVT patients with low PS activity

We measured PC and PS activity in 34 out of 163 DVT patients. We found that 11 patients showed PS activity of less than 50% with PC activity of more than 90%, suggesting functional low PS activity, where the patient with the large *PROS1* deletion was included. Among 11 patients with low PS activity, five carried causative point mutations including missense or splice site mutations (manuscript in preparation by YS and TM). Therefore, in our Japanese DVT patient group, one patient with a large *PROS1* deletion was identified in six DVT patients with low PS activity and no causative point mutations in *PROS1*.

Discussion

With a few exceptions, gene deletion measurements are not routine in most mutation screening studies, and thus alterations of gene deletion on the kilobase scale are thought to be underestimated. By MLPA, Q-PCR, and direct sequencing analysis of the *PROS1* coding regions, in addition to genotyping of the microsatellites and SNP markers flanking *PROS1*, we detected a large deletion encompassing at least the whole *PROS1* coding regions in 1 out of 163 Japanese patients with DVT.

Evaluation of the deletion detection methods

The presence of the highly homologous pseudogene (>96%) *PROSP* hampered the analysis of the gene deletion in *PROS1* by the conventional techniques. In the present study, therefore, we used the MLPA method to detect the deletions in *PROS1*. The accuracy of this method was confirmed by three rounds of MLPA and validated directly by Q-PCR. The presence and possible range of the deletion was further confirmed by direct sequencing of *PROS1* as well as microsatellite analysis and SNP linkage analysis of the flanking regions of *PROS1*. With the sequencing of *PROS1* exon regions, the loss of heterozygosity of all the SNPs of *PROS1* indicates the existence of a hemizygous deletion encompassing the whole *PROS1* gene in one DVT patient. The boundaries of the deletion were deduced by allele zygosity analysis of the SNPs and microsatellite markers centered on *PROS1*. The marker density selected for analysis also has an impact on the accuracy of discriminating the range of the deletion. In the present study, the breakpoints of the deletion could only be limited to the range between D3S3619, which was detected as the first heterozygous microsatellite in the region upstream of *PROS1*, and the centromere located downstream of *PROS1*. To further define the range of the deletion, it will be necessary to determine the segregation pattern of the investigated SNP and microsatellite markers in other affected individuals of the same family (18).

Nature of the large deletion of *PROS1*

The large deletion in the present study was defined as ranging at a minimum distance of 107 kb between the *PROS1* promoter and

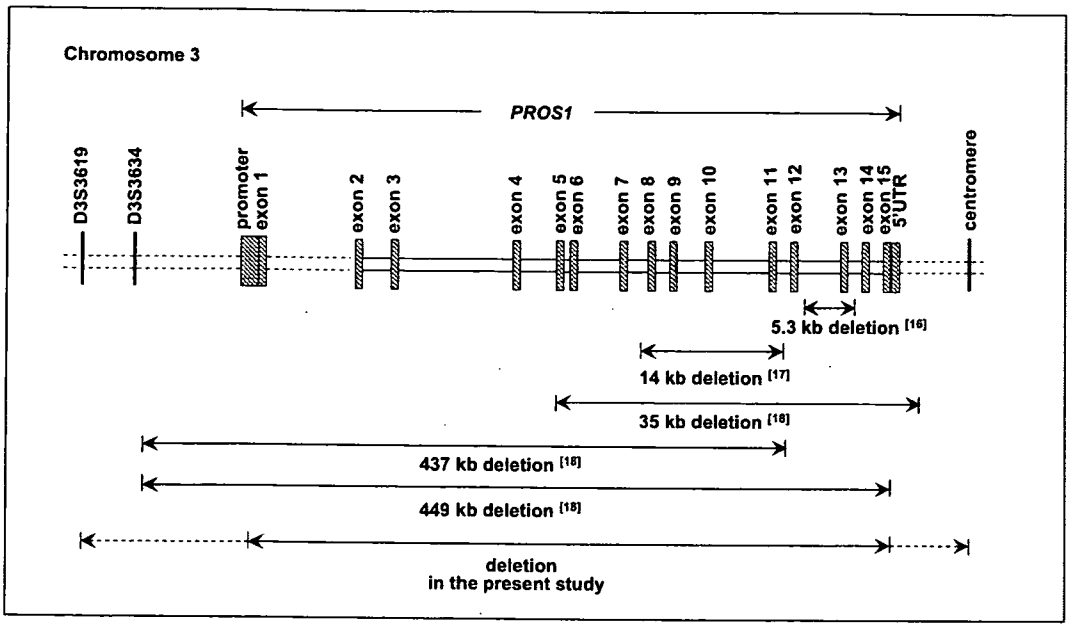

Figure 4: Outline of reported large deletions encompassing *PROS1* on chromosome 3. The location of centromere, *PROS1*, and two upstream microsatellite markers (D3S3619 and D3S3634) are sketched on chromosome 3. The relative long distance between exons 1 and 2 is represented by the dotted line. The distance of the regions flanking *PROS1* is represented by the dotted line. The minimum range of the deletion in the present study is marked with the solid line and the flanking maximum range is marked with the dotted line. The reported deletions are taken from the references (16–18).

exon 15 MLPA probes and at a maximum distance from the centromere to before the D3S3619 marker, the first heterozygous marker in the upstream of *PROS1* in chromosome 3. To date, a total of five large *PROS1* deletions have been described (Fig. 4) (16–18). A 5.3 kb deletion spanning over 90% of intron 12, the entire exon 13, and about a quarter of intron 13, was detected in two *PROS1*-deficient families by Southern hybridization (16). A long-PCR-based technique identified a deletion from intron 7 to 11 that removed approximately 14 kb (17). With a dense set of SNP and microsatellite markers, three large deletions that encompassed at least 35 kb including exons 5–15, 437 kb including exons 1–11, and 449 kb including the whole *PROS1* gene were identified (18). The deletion in the present study was another one with the definite minimum range encompassing the whole *PROS1* gene.

Large deletion of *PROS1* and PS activity

The free PS antigen level and PS activity of the patient with the deletion in *PROS1* were 2.1 μg/ml (normal range: 6.5–9.8 μg/ml) and 16% (normal range: 65–105%), respectively, so that, in addition to the hemi-deletion of the *PROS1* gene, other genetic variations could influence the levels of the PS antigen and activity. However, no causative point mutations were found in this patient by DNA sequencing. If we take the deletion as the maximum, the deletion may encompass four other genes, *ARL13B, STX19, DHFRL1*, and *NSUN3* (Fig. 3). To our knowledge, none of these genes are known to be associated with thrombosis, and thus their absence is unlikely to affect the PS deficiency phenotype. The four genes were also deleted in other patients with PS deficiency (18). Since the characteristics of these genes and the corresponding phenotypes have not yet been clarified, quantitative analysis of these genes was not considered in the present study. Another genetic variation that was missing by our analysis was the existence of chromosomal rearrangements such as an inversion of a large segment of DNA which has been found to be a major genetic defect in the factor VIII gene in patients with severe hemophilia A (30). Mutations in the promoter region might be another possible explanation for the low PS activity. The *PROS1* promoter region has recently been characterized and liver-specific cis-acting DNA elements have been identified, but the role of this region in PS deficiency is still unknown (31).

Mechanism of the large deletion

We speculated that the deletion might occur by non-allelic homologous recombination which can generate rearrangements as a result of recombination between highly similar duplicated sequences, such as segmental duplications (32). It has been confirmed that 24% of the 1,447 copy-number variations in the human genome are associated with segmental duplications. Segmental duplications were more frequently associated with the long copy-number variations (33). However, the segmental duplications were not present in the vicinity of the *PROS1* gene at the pericentromeric region in chromosome 3 (34). Regardless of the recombination mechanism, genomic architectural features have been associated with many rearrangement breakpoints (35). This suggests that the large deletion in *PROS1* is not a random event, but might result from a predisposition to rearrangement due to a complex genomic architecture that may create instability in the genome.

Large *PROS1* deletion in Japanese DVT patients

All of the large deletions of *PROS1* so far reported were detected in affected individuals in PS deficiency families (15–18). In the present study, a *PROS1* deletion was identified in a patient with DVT, but unfortunately, the family members were not available for analysis. The presence of a large deletion in one out of the 163 DVT patients suggested that a large deletion of *PROS1* is relatively rare in DVT patients. However, if the deletion was obtained in specified patients with low PS activity (<50%) and normal PC activity (>90%), it was found in one out of 11 patients. When we looked at DVT patients with low protein S activity who did not carry the causative point mutations in *PROS1*, one out of

six patients had a large *PROS1* deletion. Large deletions in *PROS1* have been previously identified by segregation analysis in three out of eight *PROS1* causative point mutation-negative families with PS deficiency (18). Large deletions of *PROS1* might be more common in familial PS deficiency than previously thought, and MLPA analysis is an effective tool to detect the deletion in *PROS1*.

In conclusion, a large deletion encompassing the whole *PROS1* gene was detected in one DVT patient with 16% PS activity. The large deletion in *PROS1* partly accounted for the low PS activity in patients with DVT. Screening for large deletions in *PROS1* might be warranted in causative point mutation-negative DVT patients with PS deficiency.

Acknowledgements

We would like to thank Dr. Masashi Akiyama and Ms. Junko Ishikawa for their technical assistance.

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Protein tyrosine kinase 2β as a candidate gene for hypertension

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Protein tyrosine kinase 2β (PTK2B) is a member of the focal adhesion kinase family and is activated by angiotensin II through Ca^{2+}-dependent pathways. An evidence exists that PTK2B is involved in cell growth, vascular contraction, inflammatory responses, and salt and water retention through activation of the angiotensin II type 1 receptor. To examine the contribution of PTK2B, we sequenced the *PTK2B* gene using 48 patients with hypertension, identified 62 genetic polymorphisms, and genotyped six representative single nucleotide polymorphisms in population-based case–control samples from 3655 Japanese individuals (1520 patients with hypertension and 2135 controls). Multivariate logistic regression analysis after adjustments for age, body mass index, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) showed −22A>G to have an association with hypertension in men (AA vs. AG + GG: odds ratio = 1.27; 95% confidence interval: 1.02–1.57; *P* = 0.030). Another polymorphism, 53484A>C (K838T), in linkage disequilibrium with −22A>G showed a marginal association with hypertension in men (AA vs. AC + CC: odds ratio = 1.25; 95% confidence interval: 0.99–1.57; *P* = 0.059). Diastolic blood pressure was 1.6 mmHg higher in men with the AC + CC genotype of 53484A>C than those with the AA genotype (*P* = 0.003), after adjustments for the same factors. These polymorphisms are in linkage disequilibrium with others in a range of 113 kb in *PTK2B*. The intracellular distribution of the recombinant PTK2B protein and that of the mutant protein with T838 were indistinguishable even after angiotensin II stimulation, both proteins localizing at a focal point in the peripheral area in the cells. Thus, a haplotype in *PTK2B* may play a role in essential hypertension in Japanese. *Pharmacogenetics and Genomics* 17:931–939

Pharmacogenetics and Genomics 2007, 17:931–939

Keywords: focal adhesion kinase 2, genetic variation, hypertension, protein tyrosine kinase 2β

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Received 27 December 2006 Accepted 26 June 2007

Introduction

Angiotensin II (Ang II) is a multifunctional hormone that regulates the functions of cardiovascular cells through intracellular signaling events initiated via interaction with cell surface Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors [1,2]. Ang II was initially described as a vasoconstrictor. Recent studies, however, demonstrate that Ang II has growth factor and cytokine-like properties. The multiple actions of Ang II are mediated by specific intracellular signaling pathways that are stimulated following initial binding of the peptide to its specific receptors. In the vasculature, AT1 receptors are mainly present in vascular smooth muscle cells (VSMCs) [3]. In the heart, AT1 receptors are expressed in cardiomyocytes and fibroblasts. AT1 receptors mediate most of the physiological actions of Ang II.

Protein-tyrosine kinase 2β (PTK2B), also known as proline-rich tyrosine kinase-2, focal adhesion kinase 2 (FAK2), cell-associated kinase β, related focal tyrosine kinase or calcium-dependent tyrosine kinase, exhibits a considerable level of structural homology to FAK, a nonreceptor tyrosine kinase which targets sites of integrin clustering [4]. Unlike FAK, PTK2B is expressed in a highly cell type and tissue-specific manner. PTK2B is activated by phosphorylation on tyrosine residues in response to various stimuli, depending on the cell type, including G protein-coupled receptor agonists (such as Ang II, thrombin, and lysophosphatidylcholine) and cellular stress (from ultraviolet irradiation, tumor necrosis factor-α, hyperosmotic shock, etc.) [5]. Activation of PTK2B requires intracellular calcium release [6]. In contrast to FAK, which is localized to adhesion plaques at the basal side of the cell, PTK2B is localized in the cytosol but can be recruited to plasma membrane, the perinuclear region, or the nucleus in response to different stimuli [7].

PTK2B is very similar to FAK, containing a kinase domain and two proline-rich domains, as well as several phosphorylated residues including an autophosphorylation site (T402), sites involved in kinase activation (T579, T580),

and a site (T881) homologous to the Grb2-binding site in FAK [6,8].

AT1 receptors activate PTK2B in a calcium-dependent manner. As PTK2B may act to regulate c-Src and to link G protein-coupled vasoconstrictor receptors with protein kinase-mediated contractile, migratory, and growth responses, it may be a potential point of convergence between Ca^{2+}-dependent signaling pathways and protein kinase pathways in VSMCs. Thus, PTK2B may play a role in hypertension through AT1 receptors.

In this study, we attempted to evaluate the *PTK2B* gene in relation to hypertension using population-based case–control samples from 3655 Japanese individuals (1520 patients with hypertension and 2135 controls). First we identified genetic variations, mainly single nucleotide polymorphisms (SNPs), in all exons of *PTK2B*. Next, we examined the association of SNPs with the presence of hypertension in the Japanese general population. Finally, we examined the intracellular localization of a mutant PTK2B with the missense mutation K838T.

Methods

Participants of the study population

The selection criteria and design of the Suita study were described previously [9,10]. Briefly, the participants had been selected randomly from the municipal population registry and stratified based on sex and age (stratified in 10-year intervals). They were all invited, by letter, to receive medical and behavioral examinations every 2 years at the Division of Preventive Cardiology, National Cardiovascular Center, Japan. DNA from the leukocytes was collected from participants who visited the National Cardiovascular Center. In this study, 3655 individuals including 1520 patients with hypertension (779 men, 741 women) and 2135 controls (930 men, 1205 women) were genotyped. All of the participants were Japanese. For DNA sequencing, 48 Japanese patients with essential hypertension at the Division of Hypertension and Nephrology, National Cardiovascular Center, Japan, were recruited. Only those who gave their written informed consent for genetic analyses were included in this study. The study protocol was approved by the Ethical Review Committee of the National Cardiovascular Center.

Measurements

Blood pressure was measured after at least 10 min of rest in a sitting position. Systolic and diastolic blood pressures (SBP and DBP) were the means of two measurements (recorded > 3 min apart). In this study, two criteria were used to define hypertension. SBP of $\geq$ 140 mmHg and/or DBP of $\geq$ 90 mmHg, or the current use of antihypertensive medication. To exclude marginal hypertension, hypertension was defined as SBP of $\geq$ 160 mmHg and/or DBP of $\geq$ 95 mmHg, or the current use of antihypertensive medication. Diabetes mellitus was defined as a fasting plasma glucose level $\geq$ 7.0 mmol/l (126 mg/dl) or nonfasting plasma glucose level $\geq$ 11.1 mmol/l (200 mg/dl) or the taking of antidiabetic medication or HbA1c $\geq$ 6.5%. Hyperlipidemia was defined as a total cholesterol concentration $\geq$ 5.68 mmol/l (220 mg/dl) or the taking of antihyperlipidemia medication. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

Blood samples drawn from the participants after 12 h of fasting were collected in ethylenediaminetetraacetate-containing tubes. Total cholesterol and high-density lipoprotein cholesterol levels were measured with an autoanalyzer (Toshiba TBA-80, Tokyo, Japan) in accordance with the Lipid Standardization Program of the US Centers for Disease Control and Prevention through the Osaka Medical Center for Health Science and Promotion, Japan.

Direct sequencing for discovering polymorphisms and genotyping of single nucleotide polymorphisms

We sequenced the entire coding region of *PTK2B* in 48 hypertensive samples in which hypertension susceptive polymorphisms would be much concentrated. The methods of direct sequencing were described previously [11,12]. SNPs having a minor allele frequency of greater than 5% were candidates for genotyping using the TaqMan-polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, California, USA) [13,14]. As a consequence, we genotyped six SNPs in the population-based samples.

Linkage disequilibrium and single nucleotide polymorphism blocks in the *PTK2B* gene

SNP genotype data for the Japanese population were downloaded from the International HapMap Consortium (*www.hapmap.org*) [15]. Positions of SNP sites were renumbered by NCBI human chromosome sequences (build 35). Pair-wise D' and LOD values were calculated and SNP blocks were inferred by Haploview [16] using its default setting parameters.

Expression of wild-type and mutant rat PTK2B

PTK2B is a rat ortholog of human PTK2B. PTK2B cDNA was inserted into an EGFP-tagging mammalian expression vector, pEGFP-C1 (BD Biosciences, San Jose, California, USA). A missense mutation, K838T, was introduced into PTK2BcDNA by site-directed mutagenesis using PCR. The mutation was confirmed by sequencing. Human umbilical vascular endothelial cells (HUVECs) were cultured in HuMedia-2 (Kurabo, Osaka, Japan) supplemented with a growth additive set and used for experiments before passage 7. HUVECs cultured on glass-bottom dishes transfected with either pEGFP-wild-type PTK2B or pEGFP-mutant PTK2B using FuGene6 (Roche Diagnostics, Basel, Switzerland) were imaged under a fluorescence microscope (IX-81, Olympus,

Tokyo, Japan). Furthermore, both wild-type and mutant cells were incubated with vehicle or 1 μmol/l Ang II (Sigma, St Louis, Missouri, USA) for 5 min to investigate the difference of cell maturity.

Statistical analysis

Student's *t*-test was used to compare mean values between groups. Frequencies were compared by χ^2 analysis. The relationships in men and women between genotypes and the presence of hypertensives were expressed in terms of odds ratios (ORs) adjusted for possible confounding effects including age, BMI, antihypertensive drug use, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) by logistic regression analysis. For multivariate risk predictors, adjusted ORs were given with 95% confidence intervals (CIs).

Association-based analyses in each sex of genotypes with blood pressures were investigated through analysis of covariance considering potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication.

Statistical analyses were performed with SAS statistical software (release 6.12, SAS Institute Inc., Cary, North Carolina, USA). The linkage disequilibrium (LD) of genotyped SNPs was calculated by using SNPAlyze version 2.1 (DYNACOM Co., Ltd, Mohara, Japan).

Results

Basic characteristics of participants of the study population

The characteristics of the 3655 participants (1709 men, 1946 women) are summarized in Table 1a. Age, SBP, DBP, BMI, percentage that are current smokers, percentage that are current drinkers, prevalence of hypertension, and prevalence of diabetes mellitus were significantly higher in men than in women. Total cholesterol, high-density lipoprotein cholesterol, and percentage that have hyperlipidemia were significantly higher in women than in men. Table 1b shows patients characteristics divided by two criteria of hypertension. Age, BMI, and percentage that are current drinkers, have diabetes and have hyperlipidemia were higher in hypertensive patients than normotensives for both criteria.

Polymorphisms in *PTK2B* and genotyping of single nucleotide polymorphisms

We sequenced 96 alleles from 48 Japanese patients with hypertension, and identified 62 polymorphisms, including four nonsynonymous and 11 synonymous SNPs (Table 2). The four nonsynonymous SNPs, 45344G > A, 48255A > G, 48273G > A, and 53484A > C, encode for the missense mutation R698H with a minor allele frequency of 0.010,

Table 1a Basic characteristics of the participants

	Women (n=1946)	Men (n=1709)
Age (year)	63.5±11.1	66.1±11.3*
Systolic blood pressure (mmHg)	128.3±19.8	130.8±19.1*
Diastolic blood pressure (mmHg)	76.5±9.7	79.2±10.3*
Body mass index (kg/m^2)	22.4±3.2	23.3±3.0*
Total cholesterol (mg/dl)	216.1±31.3*	198.7±31.4
HDL cholesterol (mg/dl)	64.9±15.2*	54.9±14.3
Current smokers (%)	6.0	30.0†
Current drinkers (%)	27.3	66.9†
Present illness (%)		
Hypertension	38.1	45.6†
Hyperlipidemia	54.9†	31.5
Diabetes mellitus	6.1	13.0†

Values are the mean±SD or a percentage. Hypertension indicates a systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or antihypertensive medication; hyperlipidemia, total cholesterol ≥ 5.68 mmol/l (220 mg/dl) or antihyperlipidemia medication; diabetes, fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or nonfasting plasma glucose ≥ 11.1 mmol/l (200 mg/dl) or antidiabetic medication. HDL, high-density lipoprotein.
*P<0.05 between women and men by the Student's *t*-test.
†P<0.05 between women and men by the χ^2 test.

Table 1b Characteristics of the patients divided by two definitions of hypertension

	NT1 (n=2135)	HT1 (n=1520)	NT2 (n=2557)	HT2 (n=1098)
Age (year)	61.8±11.6	68.8±9.4*	62.7±11.4	69.5±9.4*
Sex (F/M)	1205/930	741/779+	1426/1131	520/578+
Body mass index (kg/m^2)	22.3±3.0	23.6±3.2*	22.4±3.0	23.8±3.2*
Systolic blood pressure (mmHg)	118.0±12.0	145.6±16.5*	122.6±15.3	145.5±18.9*
Diastolic blood pressure (mmHg)	73.9±8.3	83.2±9.8*	75.6±9.0	82.9±10.5*
Total cholesterol (mg/dl)	208.3±32.8	207.5±32.2	208.7±32.5*	206.2±32.5
HDL cholesterol (mg/dl)	61.3±15.8*	58.7±15.2	61.1±15.8*	58.1±14.9
Current smokers (%)	19.3+	14.2	18.9+	13.4
Current drinkers (%)	43.6	49.0+	44.3	49.3+
Present illness (%)				
Diabetes mellitus	6.9	12.8+	7.4	13.8+
Hyperlipidemia	40.6	48.6+	41.8	49.1+

Values are the mean±SD or a percentage. HDL, high-density lipoprotein; HT, hypertension; HT1, hypertension indicates a systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg or antihypertensive medication; HT2, hypertension indicates a systolic blood pressure >160 mmHg and/or diastolic blood pressure >95 mmHg or antihypertensive medication; hyperlipidemia, total cholesterol ≥ 5.68 mmol/l (220 mg/dl) or antihyperlipidemia medication; diabetes mellitus, fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or nonfasting plasma glucose ≥ 11.1 mmol/l (200 mg/dl) or antidiabetic medication; NT, normotension.
*P<0.05 between cases and controls by the Student's *t*-test.
+P<0.05 between cases and controls by the χ^2 test.

Table 2 List of polymorphisms and their allele frequency in *PTK2B* identified by direct sequencing in 48 hypertensive patients

SNP	LD	Amino acid substitution	Region	Allele 1 frequency	Allele 2 frequency	Flanking sequence	Typing	dbSNP ID
-86282C>A			Promoter	0.870	0.130	cccggctgccaa[c/a]gcccgcgacccg	Taqman	rs7006183
-86255C>T	a		Promoter	0.924	0.076	tgctgggaatcg[c/t]ccagtcccttcc		rs12679503
-86253C>T			Promoter	0.989	0.011	ctgggaatcgcc[c/t]agtcccttcccc		
-86200G>A	b		Promoter	0.967	0.033	caatcgtgcggg[g/a]gggatggcgagg		
-86188G>A	b		Promoter	0.967	0.033	ggggatggcgag[g/a]gggagg-gagggg		
-86141G>A	c		Promoter	0.761	0.239	cttccggtgtgc[g/a]cgggaaatcttg	Taqman	rs7005244
-85972A>T	c		5′-UTR	0.771	0.229	AAAGGAGCCTCT[A/T] CCTTAACCAATC		rs6988218
-85868C>T	a		Intron 1	0.927	0.073	GCACCgtgagtg[c/t]aatcaccactta		rs12679570
-75144G>A	d		5′-UTR	0.979	0.021	TTGTGAAGACAA[G/A] CTAGACGGCAGA		
-74037T>C	c		Intron 5	0.719	0.281	cattattgcaac[t/c]tatgccatatgg		
-99G>A	e,g,i,k,m		Intron 6	0.589	0.411	gctgtccctggg[g/a]ccatgaggtatg		
-22A>G	e,g,i,k,m		5′-UTR	0.589	0.411	TGCAATGTGCCG[A/G] TCTTAGCTGCTG	Taqman	rs2241649
27T>C	e,g,i,k,m	S9S	Exon 7	0.589	0.411	CGAGCCCCTGAG[T/C] CGAGTAAAGTTG		rs1045510
45G>A	e,g,i,k,m	T15T	Exon 7	0.589	0.411	AAAGTTGGGCAC[G/A] TTACGCCGGCCT		rs1045511
162A>G	e,g,i,k,m	K54K	Exon 7	0.589	0.411	CAATCCTGGGAA[A/G] AACTTCAAACTG		rs1045512
224C>T	e,g,i,k,m		Intron 7	0.589	0.411	tgaagtgtctgc[c/t]ctgtccatctgt		rs2241650
22313G>A		E63E	Exon 8	0.990	0.010	tcctctgcagGA[g/a] ATCATCACCTCC		
22436G>A	f	T110T	Exon 8	0.875	0.125	CCCACAGATGAC[G/A] GTGGGTGAGGTG	Taqman	rs1030526
24604G>A	e,g,h,k,l		Intron 9	0.427	0.573	ttgtttgtggtg[g/a]ggtgggtggctg		rs2241652
32896T>A			Intron 12	0.865	0.135	gagtgtaaggga[t/a]gaggctggggct	Taqman	rs2241653
32932T>C	f		Intron 12	0.885	0.115	gagaagccaggg[t/c]atctgcgcggcc		rs7827965
33213T>C			Intron 12	0.927	0.073	agcagtgggcag[t/c]ctctcagcgaga		
33938C>T			Intron 14	0.957	0.043	gggaggtcgtcc[c/t]ctctctgctgcc		
34834T>C			Intron 15	0.979	0.021	tataatggcaga[t/c]tgggagctcttg		rs2303881
34862T>C			Intron 15	0.979	0.021	agaccaaaagtc[t/c]gtgacacacagg		rs2303882
36097G>A			Intron 16	0.979	0.021	ccacagcccagc[g/a]ggaagcttccag		
36456T>C	g,h,k,l		Intron 16	0.417	0.583	gtcagtcaccca[t/c]ccaggtccctgt		rs919493
36567A>G			Intron 17	0.979	0.021	acaatggggtgc[a/g]gaggacagggcc		
36648C>T	b		Intron 17	0.948	0.052	catagtttctgg[c/t]tttcaggcccag		rs12056620
38234G>T	e,i,j,k,m		Intron19	0.615	0.385	ccccgccacagc[g/t]accgtagtcaag		rs11774417
38312C>T			Intron 19	0.989	0.011	ttcctcctttat[c/t]ctcccttcgtgc		
38764C>T	b	H447H	Exon 20	0.958	0.042	CTACACAAATCA[C/T] gtgagttctagg		rs7005936
38881C>T			Intron 20	0.990	0.010	gggccccttgtc[c/t]ctaagggcctct		
38888G>A	b		Intron 20	0.958	0.042	ttgtccctaagg[g/a]cctcttgtccac		rs2241654
39431C>G	g,h,k,l		Intron 20	0.426	0.574	taggagaaaggg[c/g]cctttcctggca		rs2163176
39505G>C	b		Intron 20	0.957	0.043	agcactgggctg[g/c]accaaggggtcc		rs7005954
39722T>C	g,h,k,l		Intron 21	0.426	0.574	tggaggaggggt[t/c]cccgtcctccca		rs6996922
41359-41360delTC			Intron 23	0.990	0.010	agattcttggtc[tc/-]tttttccatctg		
42101T>C	b		Intron 25	0.958	0.042	aagacgaaactc[t/c]gtgacttattct		rs11995441
42595T>C	g,h,k,l		Intron 25	0.448	0.552	ggcgatggtgct[t/c]ctgggtgggagg		rs2241657
42977A>G	g,h,k,l		Intron 26	0.448	0.552	agggtcaaggac[a/g]ggaggctgaagc		rs2241658
45344G>A		R698H	Exon 27	0.990	0.010	AGAGGAATGCTC[G/A] CTACCGAACCCC		
46624C>G	i,j	P717P	Exon 28	0.755	0.245	ctctctccagCC[C/G] AGCCGACCTAAG		
48255A>G		M754V	Exon 29	0.989	0.011	CTCACCAGCCCT[A/G] TGGAGTATCCAT		
48273G>A	d	V760F	Exon 29	0.979	0.021	TATCCATCTCCC[G/A] TTAACTCACTGC		
48640T>A			Intron 29	0.989	0.011	ggggtaggggga[t/a]ctgtggcagctt		
53437G>A	b		Intron 30	0.957	0.043	tcttagtccttc[g/a]ctcttgtttctt		rs751018
53443G>A			Intron 30	0.989	0.011	tccttcgctctt[g/a]tttcttcctctg		
53484A>C	e,g,h,i,k,l,m	K838T	Exon 31	0.489	0.511	ATATGAATGATA[A/C] GTCCCCATTGgt	Taqman	rs751019
53748A>G	g,h,k,l,m		Intron 31	0.448	0.552	cagaaaggtcac[a/g]ttgggtcacgag		rs2251430
53860C>T	e,i,k,l,m		Intron 31	0.615	0.385	tgtctccacagc[c/t]gcatgagtgacg		rs2278319
55445A>G	g,h,k,l		Intron 32	0.448	0.552	tggtagagggga[a/g]ggggctcatttg		rs3735758
56602T>C	g,h,k,l	T876T	Exon 34	0.448	0.552	CCTGGACCGGAC[T/C] GATGACCTGGTG		rs1879184
56804-56805delCT			Intron 34	0.990	0.010	ccagcagatcct[ct/-]tagagcaagctg		
56939C>T	n		Intron 34	0.956	0.044	ctgccccttttct[c/t]cccccagAATGT		rs10093964
57034G>A	n		Intron 35	0.956	0.044	ACAGAGgtgagc[g/a]tcccattccaga		rs7007145

Table 2 (*continued*)

SNP	LD	Amino acid substitution	Region	Allele 1 frequency	Allele 2 frequency	Flanking sequence	Typing	dbSNP ID
60775A>G	g,h,k,l	A960A	Exon 36	0.435	0.565	GATGCGGCTGGC[A/G] CAGCAGAACGCC		rs1879182
60799A>G	g,h,k,l	L968L	Exon 36	0.435	0.565	CGTGACCTCCCT[A/G] AGTGAGGAGTGC		rs1879181
60835A>G		S980S	Exon 36	0.967	0.033	GCTGACGGCTTC[A/G] CACACCCTGGCT		
60926C>T			3′UTR	0.989	0.011	CCTGCAGAGTGA[C/T] GGAGGGTGGGGG		
61000T>C			3′UTR	0.989	0.011	TGCTGTTGGTCA[T/C] GTGGGTCTTCCA		
61016G>A	b		3′UTR	0.957	0.043	GGTCTTCCAGGG[G/A] GAAGGCCAAGGG		rs2271920

The A of the ATG of the initiator Met codon is denoted nucleotide + 1, as recommended by the Nomenclature Working Group (*Hum Mut* 1998; 11:1–3). The uppercase and lowercase letters are the sequence in the exon and intron region, respectively. The nucleotide sequence (GenBank Accession ID : NT_023666.16) was used as a reference sequence. The apparent linkage disequilibrium (LD), defined by an r^2 of more than 0.5, was indicated by a–m, which shows different LD group.
SNP, single nucleotide polymorphism; UTR, untranslated region; Taqman, the SNP was successfully genotyped by the Taqman method.

for M754V with a minor allele frequency of 0.011, for V760F with a minor allele frequency of 0.021, and for K838T with a minor allele frequency of 0.489, respectively. 53484A > C has been deposited in the public database with the dbSNP number, rs751019. Considering the allele frequency and the LD, we selected six SNPs for genotyping in large-scale population-based samples.

Association of single nucleotide polymorphisms with hypertension

The multivariate logistic regression analysis after adjustments for age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking) revealed that –22A > G showed an association with the presence of hypertension in men (AA vs. AG + GG: OR = 1.27; 95% CI: 1.02–1.57; $P = 0.030$). Another polymorphism, 53484A > C, accompanied by the missense mutation K838T in LD with –22A > G, showed a marginal association with the presence of hypertension in men (AA vs. AC + CC: OR = 1.25; 95% CI: 0.99–1.57; $P = 0.059$) (Table 3a). Power analyses using the SNPs with hypertension were performed. These two significant SNPs, –22A > G and 53484A > C (K838T), showed higher power, 68 and 53%, respectively. Furthermore, DBP was 1.6 mmHg higher in men with the AC + CC genotype of 53484A > C than those with the AA genotype ($P = 0.003$), after adjustments for the same factors described above (Table 4).

When hypertension was defined as SBP of > 160 mmHg and/or DBP of > 95 mmHg, or the current use of antihypertensive medication, –22A > G showed an association with the presence of hypertension in men (AA vs. AG + GG: OR = 1.38; 95% CI: 1.10–1.73; $P = 0.006$). Another polymorphism, 53484A > C (K838T), showed a significant association with the presence of hypertension in men (AA vs. AC + CC: OR = 1.31; 95% CI: 1.03–1.68; $P = 0.031$) (Table 3b). These two significant SNPs, –22A > G and 53484A > C (K838T), showed power, 90 and 68%. Taken together, *PTK2B* was associated with the presence of hypertension in men.

The pair-wise LD parameters, r^2 and D', calculated from the genotype data for these SNPs, are shown in Table 5. Two SNPs, –22A > G (rs1045510) and 53484A > C (K838T: rs751019), were in LD with an r^2 of more than 0.5. These polymorphisms showed LD extensively to make a haplotype block with more than 20 SNPs, as shown in Table 2.

To understand more about the LD in *PTK2B*, we retrieved genotype data on *PTK2B* from the public database, HapMap Project. The pair-wise D' value is shown in supplement Fig. 1. A hypertension-associated polymorphism, 53484A > C (K838T: rs751019), was in LD with rs1879181, rs1583092, rs1019832, rs4733058, rs725787, rs2322718, rs1045510, rs919495, rs11776858, rs11785606, rs10109834, and rs3735759, which are present in a stretch of 113 kb in *PTK2B*. Among the polymorphisms in LD with 53484A > C (K838T: rs751019), rs1045510 (27T > C) is a synonymous SNP encoding S9S, and the others are present in the 5′-untranslational region, in an intron, or in the 3′-untranslational region.

Expression of mutant PTK2B

As described, a haplotype of *PTK2B* including the missense mutation K838T was associated with the presence of hypertension. Figure 1 shows an amino acid sequence alignment of human, ape, dog, and mouse, in the area surrounding K838. The amino acid sequence around K838 was highly conserved among mammals, suggesting a functional role. To understand the functional roles of the K838T mutation, the rat ortholog, PTK2B, was expressed in HUVECs to see the effects on the intracellular localization of the recombinant protein. Both EGFP-tagged wild-type PTK2B and mutant PTK2B were

Table 3a Allele frequency and odds ratio of the presence of hypertension by genotypes of *PTK2B* polymorphisms by sex

SNP (allele frequency)	Genotype group	Men		Women	
		Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
−86282C>A	CC	1	0.427	1	0.074
(0.890/0.110)	CA+AA	0.90 (0.70–1.16)		1.26 (0.98–1.62)	
	CC+CA	1	0.914	1	0.181
	AA	1.05 (0.45–2.45)		1.78 (0.76–4.16)	
−86141G>A	GG	1	0.588	1	0.428
(0.671/0.329)	GA+AA	1.06 (0.86–1.30)		1.09 (0.89–1.33)	
	GG+GA	1	0.571	1	0.722
	AA	1.10 (0.79–1.55)		0.94 (0.67–1.32)	
−22A>G	AA	1	0.030	1	0.521
(0.597/0.403)	AG+GG	1.27 (1.02–1.57)		1.07 (0.87–1.32)	
	AA+AG	1	0.375	1	0.617
	GG	1.14 (0.86–1.50)		0.93 (0.70–1.24)	
22436G>A	GG	1	0.456	1	0.452
(0.820/0.180)	GA+AA	0.92 (0.74–1.14)		0.92 (0.74–1.14)	
	GG+GA	1	0.483	1	0.764
	AA	1.27 (0.65–2.45)		1.10 (0.58–2.09)	
32896T>A	TT	1	0.935	1	0.254
(0.889/0.111)	TA+AA	1.01 (0.79–1.29)		1.15 (0.90–1.48)	
	TT+TA	1	0.276	1	0.926
	AA	1.59 (0.69–3.67)		0.96 (0.37–2.50)	
53484A>C	AA	1	0.059	1	0.874
K838T	AC+CC	1.25 (0.99–1.57)		0.98 (0.79–1.22)	
(0.527/0.473)	AA+AC	1	0.600	1	0.633
	CC	1.07 (0.83–1.37)		0.94 (0.73–1.21)	

All adjusted for age, body mass index, antihypertensive drug use, present illness (hyperlipidemia, diabetes mellitus), and lifestyle (smoking and drinking). Hypertension was defined as a systolic blood pressure of >140 mmHg and/or diastolic blood pressure of >90 mmHg, or the current use of antihypertensive medication.
CI, confidence interval; SNP, single nucleotide polymorphism.

Table 3b Allele frequency and odds ratio of the presence of hypertension by genotypes of *PTK2B* polymorphisms by sex

SNP (allele frequency)	Genotype group	Men		Women	
		Odds ratio (95% CI)	*P*	Odds ratio (95% CI)	*P*
−86282C>A	CC	1	0.426	1	0.288
(0.890/0.110)	CA+AA	1.11 (0.86–1.44)		1.16 (0.88–1.52)	
	CC+CA	1	0.881	1	0.657
	AA	1.07 (0.45–2.51)		1.24 (0.48–3.15)	
−86141G>A	GG	1	0.940	1	0.100
(0.671/0.329)	GA+AA	1.01 (0.81–1.25)		1.20 (0.96–1.50)	
	GG+GA	1	0.470	1	0.305
	AA	1.14 (0.80–1.60)		0.82 (0.57–1.19)	
−22A>G	AA	1	0.006	1	0.819
(0.597/0.403)	AG+GG	1.38 (1.10–1.73)		1.03 (0.82–1.29)	
	AA+AG	1	0.067	1	0.985
	GG	1.31 (0.98–1.74)		1.00 (0.74–1.37)	
22436G>A	GG	1	0.060	1	0.490
(0.820/0.180)	GA+AA	0.80 (0.64–1.01)		0.92 (0.73–1.16)	
	GG+GA	1	0.735	1	0.360
	AA	1.13 (0.56–2.26)		1.36 (0.70–2.63)	
32896T>A	TT	1	0.420	1	0.475
(0.889/0.111)	TA+AA	1.11 (0.86–1.43)		1.10 (0.84–1.44)	
	TT+TA	1	0.691	1	0.487
	AA	1.18 (0.52–2.68)		1.42 (0.53–3.84)	
53484A>C	AA	1	0.031	1	0.554
K838T	AC+CC	1.31 (1.03–1.68)		1.07 (0.85–1.36)	
(0.527/0.473)	AA+AC	1	0.986	1	0.386
	CC	1.00 (0.77–1.30)		0.89 (0.67–1.16)	

CI, confidence interval; SNP, single nucleotide polymorphism.
All adjusted for age, body mass index, antihypertensive drug use, present illness (hyperlipidemia, diabetes mellitus), and lifestyle (smoking and drinking). Hypertension was defined as a blood pressure of >160 mmHg and/or diastolic blood pressure of >95 mmHg, or the current use of antihypertensive medication.

observed at the focal adhesions. Figure 2a and b indicated that the missense mutation, K838T, of PTK2B does not extensively alter the intracellular localization of PTK2B. As shown in Fig. 2c and d, both EGFP-tagged wild-type PTK2B and mutant PTK2B were observed at the cytosol and the immature focal adhesions without the stimulation. After Ang II stimulation, EGFP-tagged wild-type PTK2B and mutant PTK2B were partially located at the mature focal adhesions as reported previously [17] and had similar localization manner. These results indicated that the missense mutation, K838T, of PTK2B does not extensively alter the intracellular localization of PTK2B.

Table 4 Blood pressure levels by genotypes of a *PTK2B* polymorphism, 53484A>C (K838T), in men

	AA	AC	CC	*P*	AA+AC	CC	*P*	AA	AC+CC	*P*
n	459	876	366		1335	366		459	1242	
DBP	78.0±0.5	79.9±0.3	78.9±0.5	0.123	79.2±0.3	78.9±0.5	0.544	78.0±0.5	79.6±0.3	0.003
SBP	130.8±0.8	131.0±0.6	130.5±0.9	0.882	130.9±0.5	130.5±0.9	0.720	130.8±0.8	130.8±0.5	0.921

Values are mean±SD. All adjusted for age, body mass index, antihypertensive drug use, present illness (hyperlipidemia, diabetes mellitus), and lifestyle (smoking and drinking).
DBP, diastolic blood pressure; SBP, systolic blood pressure.
DBP and SBP are expressed in mmHg.

Table 5 Linkage disequilibrium of six genotyped *PTK2B* polymorphisms expressed by r^2 and absolute D'

	−86282 C>A	−86141 G>A	−22 A>G	22436 G>A	32896 T>A	53484 A>C
−86282C>A	–	0.24	0.17	0.02	0.41	0.09
−86141G>A	1.00	–	0.14	0.07	0.07	0.04
−22A>G	0.94	0.42	–	0.13	0.09	0.53
22436G>A	0.79	0.40	0.97	–	0.02	0.02
32896T>A	0.66	0.50	0.68	0.89	–	0.13
53484A>C	0.82	0.27	0.85	0.36	0.94	–

Upper right represents r^2 and lower left shows absolute D'.

Fig. 1

		*	
Human	830	DPMVYMNDKSPLTPEKEV	847
Ape	923	DPMVYMNDKSPLTPEKEV	940
Dog	830	DPMLYMNDKSPLTPEKEA	847
Mouse	830	DPMVYMNDKSPLTPEKEA	847
Rat	788	DPMVYMNDKSPLTPEKEA	805

Amino acid sequences of human, ape, dog, mouse, and rat PTKB2 are aligned. Numbers on left and right side indicate positions of amino acid residues. *K838.

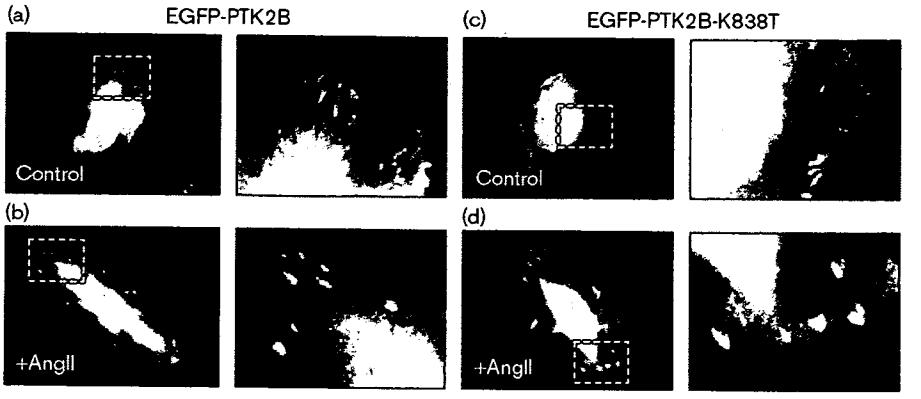
Discussion

In this study, we evaluated *PTK2B* as a candidate for a susceptibility gene for hypertension using population-based case–control samples including 3655 Japanese individuals (1520 patients with hypertension and 2135 controls). The multivariate logistic regression analysis after adjustments for confounding factors showed that −22A > G and 53484A > C (K838T) in *PTK2B* showed an association with the presence of hypertension in men. This association was stronger when hypertension was defined as SBP of ≥ 160 mmHg and/or DBP of ≥ 95 mmHg, or the current use of antihypertensive medication. Both SNPs were in LD with other polymorphisms in *PTK2B*, thus comprising an extensive haplotype block 113 kb in length. Therefore, this extensive haplotype block in *PTK2B* may be an important determinant for hypertension.

PTK2B is involved in the signaling pathways of Ang II and endothelin-1 (ET-1), two important vasoconstrictors, in cardiovascular cells [5,18,19], and nitric oxide, an important vasodilator, inhibited Ang II-induced activation of PTK2B [20]. In addition, PTK2B-mediated Ang II or ET-1-augmented migration and protein synthesis in VSMCs [17,21,22]. The augmented migration and protein synthesis by VSMCs could lead to medial thickening and progressive luminal narrowing of resistant blood vessels and result in hypertension [23,24]. Moreover, VSMCs from spontaneously hypertensive rats exhibited increased cell growth compared with those from normotensive Wistar–Kyoto rats [25], and increased PTK2B activity was involved in this effect [26]. All these results suggest that genetic variations of *PTK2B* influence the net-effects of vasoactive factors on VSMC phenotype and contribute to hypertension. Furthermore, *PTK2B* was originally identified in the human hippocampus and its mRNA was detected mainly in human brain and kidney [27]. An evidence to suggest that Ang II is a neurotransmitter and upregulation of the renin-angiotensin system in brain contributes to hypertension exists [28]. Therefore, an effect of genetic variations of *PTK2B* on the regulation of the cardiovascular system by the central nervous system is expected. Transgenic and knockout techniques for the *PTK2B* gene *in vivo* are necessary to clarify this point.

In this study, we genotyped six SNPs. Therefore, after applying the Bonferroni correction for multiple testing, the level of significance is $P < 0.0083$ (0.05/6 for 6 loci). −22A > G showed a significant association with hypertension in men ($P = 0.006$) even with use of a strict Bonferroni correction, when hypertension is defined as SBP of ≥ 160 mmHg and/or DBP of ≥ 95 mmHg, or the current use of antihypertensive medication. In addition, 53484A > C still showed a significant association with blood pressure levels in men ($P = 0.003$) after the Bonferroni correction. Power analysis also showed that these two SNPs, −22A > G and 53484A > C, had higher power more than 50%, and rest of SNPs did not have

Fig. 2



Fluorescent imaging of wild-type and mutant PTK2B molecules. HUVECs transfected with the plasmids encoding EGFP-PTK2B (a, b) and EGFP-PTK2B K838T (c, d) were starved for 4 h, and stimulated with vehicle (a, c) or 1 μmol/l Ang II for 5 min (b, d). Right side images of each panel show magnified view of the area in squares. HUVECs, human umbilical vascular endothelial cells.

power above 50%. Thus, *PTK2B* is speculated to be a susceptibility gene for hypertension.

The mechanisms by which the two SNPs (−22A > G and 53484A > C) might be associated with hypertension in men only are unknown. No association in women was observed. This inconsistency might be derived from sex differences. Regarding sex differences, the incidence and rate of progression of hypertension was markedly higher in men than in age-matched premenopausal women and, after menopause, this relationship no longer existed [29]. In various hypertensive animal models, males showed higher blood pressure levels than females owing to greater levels of Ang II-NADPH oxidase-mediated upregulation of the production of reactive oxygen species [30,31], Ang II-induced enhancement of sympathetic nerve activity [32], decreased nitric oxide production [33], and a high ratio of AT1/AT2 receptors of Ang II [34]. In addition, the elevation in blood pressure after the administration of ET-1 was much higher in male rats than in female rats [35], because estrogen might reduce ET-1-induced vasoconstriction [36]. As PTK2B is involved in the signaling pathways of Ang II and ET-1 and nitric oxide inhibits Ang II-induced activation of PTK2B [20], sex differences in the relationship between *PTK2B* polymorphisms and hypertension may be ascribed to the influences of these vasoactive factors.

The missense mutation K838T seemed to be important to the function of PTK2B. We expressed the mutant protein in mammalian cells and examined its intracellular localization by fluorescence imaging. It was clear that the mutant did not show extensive changes in terms of localization even after Ang II stimulation. This imaging, however, only looked at the localization. We have to examine the kinase activity of the mutant protein in a future study. In addition, −22A > G and SNPs in LD with 53484A > C (−99G > A, rs919495, rs11776858, rs11785606, rs10109834, and rs3735759) are present in the 5′-untranslational region. Whether they could influence *PTK2B* gene expression needs to be clarified.

In summary, we have found an association between hypertension and SNPs of *PTK2B*. Association-based studies are not consistently reproducible due to false positive results, false negative results, or true variability in associations among different populations [37]. Therefore, confirmation of the result in additional cohorts may be required.

Acknowledgements

The authors express their gratitude to Drs Otosaburo Hishikawa, Yasushi Kotani, and Katsuyuki Kawanishi and Mr Tadashi Fujikawa for their continuous support of our population survey in Suita city. They thank the members of the Satsuki–Junyukai. They also thank all the staff at the Division of Preventive Cardiology for supporting medical examinations and Yoko Tokunaga, Mariko Banno, and Chihiro Tanaka for their technical assistance. They thank Dr Naoki Mochizuki for his great help with the fluorescence microscopy. Genotype data on the Japanese population were downloaded from The International Hapmap Consortium. This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) of Japan, a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Journal of Human Hypertension (2007) 21, 883–892

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ORIGINAL ARTICLE

Association of single nucleotide polymorphisms in endothelin family genes with the progression of atherosclerosis in patients with essential hypertension

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Endothelin-1 (ET-1) is a potent vasoconstrictive peptide and its activity is mediated by the receptors ET type A (EDNRA) and ET type B (EDNRB). Although ET-1 is thought to play an important role in the development of atherosclerosis, it remains unclear whether polymorphisms of ET-1 family genes, including the ET-1 gene (*EDN1*), *EDNRA*, *EDNRB* and the genes for endothelin converting enzymes 1 and 2 (*ECE1* and *ECE2*), are associated with the progression of atherosclerosis. We investigated the relationship between 11 single nucleotide polymorphisms (SNPs) of ET-1 family genes (including three in *EDN1*, one in *EDNRA*, two in *EDNRB*, four in *ECE1* and one in *ECE2*) and atherosclerotic changes assessed using pulse wave velocity (PWV) and carotid ultrasonography in 630 patients with essential hypertension (EHT). In male subjects, we found significant differences in brachial-ankle PWV (baPWV) in additive and recessive models in *EDNRB*-rs5351 after Bonferroni correction. Also in male subjects, there were significant differences in mean intima-media thickness (IMT) in additive and recessive models in *EDNRA*-rs5333 after Bonferroni correction. We found no significant correlation between any SNPs in the ET family genes and baPWV, IMT and Plaque score (PS) in female subjects. Furthermore, after multiple logistic regression analysis, only *EDNRB*-rs5351 indicated as an independent risk of atherosclerosis in male hypertensive subjects. Of the endothelin-related genes, *EDNRB*-rs5351 was the most susceptible SNP associated with atherosclerosis in male hypertensives, and the genetic background may be involved in the progression of atherosclerosis in EHT patients.
Journal of Human Hypertension (2007) **21**, 883–892; doi:10.1038/sj.jhh.1002234; published online 24 May 2007

Keywords: endothelin 1 (ET-1) family genes; single nucleotide polymorphisms (SNPs); atherosclerosis

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide produced primarily by vascular endothelial cells and appearing in many other organs.[1] ET-1 is thought to play an important role in the development of atherosclerosis through endothelial dysfunction and the proliferation of vascular smooth muscle cells (VSMCs). ET-1 may be a marker for arterial vascular disease; Lerman *et al.*[2] showed a significant correlation between plasma endothelin levels and the number of vascular disease sites. Some reports have linked plasma levels of ET-1 to hypertension, while others have argued against this relationship. Hirai *et al.*[3] suggested that high ET-1 levels are not related to hypertension, but rather to subclinical renal dysfunction and smoking. The expression of ET-1 is mediated by the activation of specific receptors: ET type A (EDNRA) and ET type B (EDNRB). The former is the predominant ET receptor on VSMCs, and signalling via EDNRA causes long-lasting vasoconstriction.[4,5] EDNRB is located primarily on endothelial cells and its signalling promotes the formation of nitric oxide, as well as the clearance and reuptake of ET-1.[6–9] Endogenous ET-1, which acts via EDNRA, increases resistance-vessel tone in subjects with hypertension to a level greater than that in smokers and in subjects with hypercholesterolemia.[10] Plasma ET-1

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Received 14 March 2007; revised 19 April 2007; accepted 25 April 2007; published online 24 May 2007

concentrations can be reduced by resistance training and aerobic exercise.[11]

Pulse wave velocity (PWV) is generally recognized as a surrogate marker for atherosclerosis.[12] Using sheep, McEniery *et al.*[13] showed that endogenous ET-1 production regulates large artery PWV *in vivo*. They also revealed that exogenous ET-1 increases PWV and that this increase can be blocked by ET type A receptor blockers. Vuurmans *et al.*[14] examined whether ET-1 increases central aortic systolic blood pressure, pulse pressure and PWV in healthy men, and the effect of ET-1 is prevented by ET-1 receptor blockers.

It remains unclear, however, whether gene polymorphisms of the ET-1 family (including the ET-1 gene (*EDN1*), *EDNRA*, *EDNRB* and the genes for endothelin converting enzymes 1 and 2 (*ECE1* and *ECE2*) are associated with the progression of atherosclerosis. Therefore, we investigated the relationship between single nucleotide polymorphisms (SNPs) of ET-1 family genes and atherosclerotic changes assessed by PWV and carotid echo ultrasonography in patients with essential hypertension (EHT).

Materials and methods

Subjects

This study included 630 outpatients (340 men and 290 women) with EHT at the Division of Hypertension and Nephrology of the National Cardiovascular Centre (NCVC). All subjects provided written informed consent and the protocol was approved by the ethics committee of NCVC. Hypertension was defined as a systolic blood pressure (SBP) of 140 mm Hg or greater and/or a diastolic blood pressure (DBP) of 90 mm Hg or greater, or the current use of antihypertensive medication. The blood pressure used was the average of at least three measurements made during each visit. We also measured brachial-ankle PWV (baPWV) using Form ABI (Colin Medical Technology) and examined carotid arteries using a commercially available ultrasound system (SSA-390A; Toshiba Medical, Japan).[4] We measured the mean intima-media thickness (IMT) and maximum-IMT (max-IMT) of common carotid arteries and the sum of the plaque score (PS) of bilateral common and internal carotid arteries, as reported previously.[15] Blood samples were also taken at the clinic, and diabetes mellitus was defined as a fasting blood sugar level greater than 126 mg/dl, an HbA_{1c} level greater than 6.5%, or the use of anti-hyperglycemic medications. Hyperlipidemia was defined as a total-cholesterol concentration of 220 mg/dl or greater, a triglyceride (TG) concentration of 150 mg/dl or greater, or the use of lipid-lowering medication at the time of the first examination. Subjects who had ankle-brachial indices (ABI) lower than 0.9 were excluded because their baPWV readings were unreliable.

Table 1 The entire coding region of the endothelin-1 gene

		Allele 1/Allele 2			*Allele 1*		*Allele 2*		*Allele frequency*			
Gene name	*Locus*	*SNPs*	*Aa info.*	*Region*	*Homo*	*Hetero*	*Homo*	*Total*	*Allele 1*	*Allele 2*	*Flanking sequence*	*dbSNP ID*
EDN1	6p24-p23	10bp del.(−173)		promoter	47	1	0	48	0.990	0.010	AGGTTTAGCAAA[GGTCTCTAAT/-]GGGTATTTTCTT	
		A201- (4A/3A)	5′-UTR	exon1	1	8	39	48	0.104	0.896	TTTCTCCCCGTT[A/-]AAAGGGCACTTG	
		G2087A	Gly36Arg	exon2	47	1	0	48	0.990	0.010	GGTGAGAACGGC[G/A]GGGAGAAACCCA	
		G2244T		intron2	8	18	21	47	0.362	0.638	ATTGTAACCCTA[G/T]TCATTCATTAGC	rs2070699
		T2252A		intron2	46	1	0	47	0.989	0.011	CCTAGTCATTCA[T/A]TAGCGCTGGCTC	
		T3609C		intron2	33	12	2	47	0.830	0.170	AAGACTATTAAT[T/C]ACACTAATATAG	rs1800543
		A3730G	Glu106Glu	exon3	0	1	46	47	0.011	0.989	AACAGACCGTGA[A/G]AATAGATGCCAA	rs 5369
		T5629A		intron4	47	1	0	48	0.990	0.010	GGGTGATTTTTT[T/A]AAAATAACATTT	
		G5727T	Lys198Asn	exon5	31	14	2	47	0.809	0.191	GCTGAAAGGCAA[G/T]CCCTCCAGAGAG	rs 5370

Abbreviation: SNPs, single nucleotide polymorphisms.
By Gene Cards. Version: 2.25, released 3 July, 2002.

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