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POLYMORPHISMS IN THE 5′-UTR OF *PTEN* AND OTHER GENE POLYMORPHISMS IN NORMAL JAPANESE INDIVIDUALS

Polymorphisms are distributed differently in populations, including those of regions, ethnic groups, and diseased patients. In order to investigate variation in nucleotide sequences in normal individuals, we isolated genomic DNA from the blood of healthy Japanese individuals and sequenced the 5′-untranslated region (5′-UTR) of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene and the gene promoter, intron, and exon nucleotides of p53, $p14^{ARF}$, murine double minute 2 (MDM2), and the β_2- and β_3-adrenoceptor (-AR). We found polymorphisms in these regions, including a deletion at positions −465 to −463 and a substitution at position −404 in PTEN and a substitution at position −4924 in $p14^{ARF}$, in normal individuals. Individuals with or without the PTEN polymorphism harbored a different distribution of polymorphisms, including simultaneous alterations in nucleotides of p53, MDM2, and β_3-AR, and also harbored some polymorphic nucleotides located in the same set of associatively altered nucleotides. Our results show that multiple nucleotides, including the PTEN nucleotides, are altered in normal Japanese individuals and provide useful information for genotyping studies in individuals and populations.

Introduction. Control subjects harbor polymorphisms of the phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) gene, including those at positions −1026, −903 [1], and −9 [2] in the 5′-untranslated region (5′-UTR). The *PTEN* −1026 and −903 polymorphisms are located around the binding sites of transcription factors such as Sp1 [1], and the −9 polymorphism is located close to a Kozak sequence. A polymorphism of the *p53* gene in codon 72, which is commonly found in various populations, including the Japanese population [3], is distributed latitude-dependently among resident populations of eastern Asia in areas located from 10−50° north; the distribution of this polymorphism is also associated with winter temperatures [4]. In addition, an intron polymorphism at position SNP309 of the murine double minute 2 (*MDM2*) gene shows a different distribution among regions that are exposed to distinct levels of ultraviolet radiation [4]. A *PTEN* promoter polymorphism at position −1142, which is found in normal Japanese individuals, is located within a p53-binding element [5], and a *p53* promoter polymorphism at the −824 to −818 poly(C) positions (a C-to-C insertion polymorphism) found in a normal Japanese population [6] and the *PTEN* −1142 promoter polymorphism alter cellular transcriptional activity in the presence or absence of serum in culture medium [5]. *PTEN* gene expression is increased at 32 °C in cells expressing a temperature-sensitive p53 mutant [7] and differs among individuals, including control subjects [1] and those with non-diseased tissues [8, 9].

Polymorphisms are reportedly associated with individual predispositions, including those to diseases. A population-based study using polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) and direct nucleotide sequencing analyses has shown that the −9 polymorphism in the *PTEN* 5′-UTR is associated with a disease of diabetes exhibiting metabolic abnormalities in a Japanese population [2]. A similar association with diabetes has also been reported for other gene polymorphisms, including a $p14^{ARF}$ polymorphism at position −3735, in a European population [10]. Some promoter polymorphisms within the $p14^{ARF}$ 4.5 kb promoter, including the −3735 polymorphism, have been observed in an Asian (Korean) population [11]. Further, other association studies based on po-

pulations or follow-up observations have shown that exon 1 polymorphisms of the β_2- and β_3-adrenoceptor(-AR) genes at codons 27 and 64 are associated with not only metabolic diseases, such as diabetes and obesity [12, 13], but also other diseases, such as hypertension [14] and cancers of the breast [15] and colon [16]. In such association studies, polymorphisms are observed in control subjects who do not suffer from disease and are not necessarily observed in all of the patients.

Individuals, including non-cancer patients and normal individuals, harbor multiple nucleotide alterations in each gene; e.g., alterations in intron 1, 3, 4, and 8 of the *PTEN* gene [17] or in exon 1 at position −628 and intron 1 at positions −466 and −215 (SNP309) of the *MDM2* gene [6]. The *MDM2* −628 and −466 polymorphisms observed in normal Japanese individuals are included in associative alterations in nucleotides [6], and the *PTEN* intron 1, 3, 4, and 8 polymorphisms observed in individuals, including non-cancer patients, are involved in a highly linked sequence of nucleotides [17]. The SNP309 polymorphism of *MDM2* is reportedly in linkage disequilibrium with other polymorphisms of *MDM2* and is distributed differently between ethnic groups [18]. Similar linkage has also been shown for the $p14^{ARF}$ promoter polymorphisms in a Korean population [11]. An association study has reported that the presence of the −9 polymorphism of the *PTEN* 5′-UTR decreases the risk of some carcinomas in the absence of another *PTEN* intronic polymorphism (a sequence insertion of five nucleotides) in a Chinese population [19]. Polymorphic nucleotides linked with other polymorphisms are useful for genotyping studies in populations.

In the present study, in order to investigate whether normal individuals harbor other *PTEN* polymorphisms and whether each individual harbors multiple nucleotide alterations, we isolated genomic DNA from the blood of healthy Japanese individuals and sequenced the 5′-UTR of *PTEN* together with other gene promoter, intron, and exon nucleotides at positions −824 to −818 of *p53*; position −3735 of $p14^{ARF}$; positions −628, −466, and −215 (SNP309) of *MDM2*; and codons 27 and 64 of β_2- and β_3-*AR*. We also sequenced the nucleotides upstream of the $p14^{ARF}$ 4.5 kb promoter sequence and searched polymorphic nucleotides associatively altered with other $p14^{ARF}$ polymorphisms.

Materials and methods. *Study subjects and genomic DNA extraction.* Human peripheral blood was obtained from 21 healthy Japanese students (in which male students outnumber female students) in their early twenties who consented to have their DNA sequenced for identification of polymorphisms. Genomic DNA was extracted from whole blood with a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA analysis showed that the frequency of a known polymorphism (NAD(P)H:quinone oxidoreductase (NQO1) polymorphism (C609T); [20]) was similar to that previously found in control Japanese subjects (the frequency of the *NQO1* polymorphism was 48 % homozygous C/C, 38 % heterozygous C/T, and 14 % homozygous T/T). We investigated the frequency of the examined polymorphism by using samples obtained from 12 randomly selected students (the polymorphic frequency of *NQO1* was 58.3 % homozygous C/C, 33.3 % heterozygous C/T, and 8.3 % homozygous T/T), and these randomly selected samples (seven individual samples in the case of the $p14^{ARF}$ promoter) were used to identify polymorphisms.

PCR amplification. The *PTEN* 5′-UTR and the promoter sequence of $p14^{ARF}$ were amplified by PCR in a reaction mixture containing genomic DNA (0.1 μg), a primer set (Table 1), and DNA polymerase (KOD-Plus DNA polymerase ([21]; Toyobo, Osaka, Japan) for PTEN or PfuTurbo DNA polymerase ([22]; Stratagene, La Jolla, CA) for $p14^{ARF}$) according to the manufacturer's instructions. Amplification was performed in a thermal cycler (Takara PCR Thermal Cycler MP; Takara Bio, Osaka, Japan) under the following conditions: denaturation at 94 °C for 15−30 s, annealing at melting temperature [Tm] − 5 °C for 1 min, and extension at 68−72 °C for 2 min. For the promoter, intron, and exon regions of *p53* and *MDM2*, PCR amplification was performed by using a primer set (Table 1) as described previously [6]. For the exon regions of β_2- and β_3-*AR*, PCR was performed by using the following primers as described previously [15, 23]: β_2-AR (forward primer: 5′-GAATGAGGCTTCCAGGCGTC-3′; reverse primer: 5′-GGCCCATGACCAGATCAGCA-3′) and β_3-

AR (forward primer: 5′-CGCCCAATACCG-CCAACAC-3′; reverse primer: 5′-CCACCAG-GAGTCCCATCACC-3′).

Determination of nucleotide sequences and analysis for polymorphisms. DNA amplified by PCR (PCR products) was used for sequence reactions (for *PTEN*, *p14*^{ARF}, *p53*, and *MDM2*) to determine the polymorphic nucleotides. The sequence reactions were performed using an ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Biosystems, Foster City, CA, USA), and nucleotide sequences were determined using an ABI PRISM 377 automated DNA sequencer (Perkin-Elmer Biosystems). The nucleotide sequences were compared with DNA sequences (accession nos. AF067844, AF082338.1, X54156.1, and U39736.1) in the DNA database of NCBI GenBank by using the basic local alignment search tool (BLAST; [24]). For the detection of sequence alterations, nucleotides were compared among individuals by using the sequence alignment editor program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and heterozygous nucleotide sequences were analyzed in detail by changing the horizontal scale for the alignments of allelic nucleotides.

Detection of restriction fragment length polymorphisms (RFLPs) of β_2- and β_3-AR. PCR products were treated with or without a restriction enzyme (*Fnu4*HI; New England Biolabs, Inc., Ipswich, MA, USA (for β_2-AR) or *Bst*OI; Promega, Madison, WI, USA (for β_3-AR)) for 2 h at 37 °C in NEBuffer (New England Biolabs, Inc.) containing bovine serum albumin according to the

Table 1

Primers used for PCR and sequencing

Primer position[1]	Oligonucleotide primer
PTEN	
−1530 to −1507	5′-TCTGCGAACGATTGTGATCCGACA-3′
−845 to −822	5′-AGTTCTCTCCTCTCGGAAGCTGCA-3′
−605 to −628	5′-GAGGAAGAGGCTGCACGGTTAGAA-3′
−375 to −398	5′-GCCGCCGTGTTGGAGGCAGTAGAA-3′
−247 to −270	5′-AGAAGACGAATAATCCTCCGAACG-3′
−33 to −56	5′-AGAGAGATGGCAGAAGCTGCTGGT-3′
p14^{ARF}	
−5322 to −5300	5′-GTCTCCTCACAAGCATGTCAATC-3′
−5009 to −4987	5′-GTTACATACATGAGTTATAGGAA-3′
−4050 to −4028	5′-CTGGCTCTTGCTGGCCATGAAGT-3′
−4773 to −4794	5′-GGCAGCATTACAATATCTAGTC-3′
−3267 to −3289	5′-CTTCAACTGCTTGGATGAGGACC-3′
−2986 to −2965	5′-CTGGATAACGATGCTTCAGTCA-3′
−2702 to −2682	5′-TGTATATGATGTTTGCACAAC-3′
−2300 to −2279	5′-TACAGAGGCGGAGGCCGGCTGA-3
−2532 to −2553	5′-CTATATCAGTCAGTTCTCCAGG-3′
−2157 to −2178	5′-TACTTATGTACTTGTTTACTTG-3′
p53	
−918 to −899	5′-GCTGGGAGTTGTAGTCTGAA-3′
−669 to −690	5′-CATTGTTGTATTCCTGAGTGCC-3′
MDM2	
−725 to −704	5′-TCTGACCGAGATCCTGCTGCTT-3′
−310 to −289	5′-TTCGGACGGCTCTCGCGGCGGT-3′
+96 to +75	5′-AAGCTACAAGCAAGTCGGTGCT-3′

Indications. [1]Nucleotide positions are numbered by considering the position of nucleotide A at the initiation site for translation in *PTEN* (accession no. AF067844), nucleotide G [56] in *p14*^{ARF} (accession no. AF082338.1), nucleotide G [57] in *p53* (accession no. X54156.1), and nucleotide C at the 5′ end of exon 2 [58] in *MDM2* (accession no. U39736.1) as +1.

manufacturer's protocol and separated by 3 % agarose gel electrophoresis. RFLPs were detected by using ethidium bromide and ultraviolet light, and we evaluated the sizes of the DNA fragments generated by treatment with or without the restriction enzyme; *β_2-AR* Gln/Gln at codon 27 produces 174-, 97-, 55-, and 27-bp fragments and Gln/Glu at codon 27 produces 229-, 174-, 97-, 55-, and 27-bp fragments, and *β_3-AR* Trp/Trp at codon 64 produces 99-, 62-, and 30-bp fragments and Trp/Arg at codon 64 produces 161-, 99-, 62-, and 30-bp fragments.

Results. *PTEN polymorphisms and nucleotide sequence variations in normal Japanese individuals.* We sequenced the 5′-UTR upstream of the −9 position of *PTEN* and further determined other nucleotides in the promoter, intron, and exon regions of *p53*, *$p14^{ARF}$*, *MDM2*, and *β_2-* and *β_3-AR*. We detected polymorphisms in the *PTEN* 5′-UTR (Figs 1−3, look a pasting in at the end of number) and in the other examined genes (Fig. 4, look a pasting in at the end of number and Tables 2 and 3) in normal Japanese individuals, and we observed individual differences in these sequences (Tables 2 and 3). These sequence variations comprised deleted, substituted, and inserted nucleotides. In the *PTEN* 5′-UTR, we found a deletion of nucleotide G (Fig. 1) (nucleotide C in the opposite DNA strand to the 5′-UTR sequence; Fig. 2) at positions −465 and −463 and a nucleotide substitution of C to T (Fig. 1) (G to A in the opposite strand to the 5′-UTR; Fig. 3) at position −404; these polymorphisms were observed in individuals I and VIII, respectively (Table. 2). These individuals also harbored a C-to-C insertion at the −824 to −818 poly(C) positions of *p53* (Fig. A.1, http://cytgen.com/articles/4620024s.pdf) and a Trp-to-Arg substitution at codon 64 of *β_3-AR* (Fig. A.3b) together with A-to-G and C-to-T substitutions at positions −628 and −466 (Fig. A.2 a and b) or a T-to-G substitution at position −215 (SNP309) of *MDM2* (Fig. A.2c) (Table 2). A Gln-to-Glu substitution of *β_2-AR* at codon 27 (Fig. A.3a) was only observed in individual III, and this individual also harbored the *p53* poly(C) and *MDM2* SNP309 polymorphisms (Table 2). The *β_3-AR* codon 64 polymorphism and the *p53* and *MDM2* polymorphisms were simultaneously found in individuals VII and XI, in addition to I and VIII, while other individuals (II, IV−VI, IX, X, and XII) harbored a polymorphism(s) in *p53* and/or *MDM2* (Table 2). An A-to-C substitution in *$p14^{ARF}$* at position −3735 was not observed in any individuals (Table 2). The polymorphic nucleotides in *PTEN*, *p53*, *MDM2*, and *β_2-* and *β_3-AR* were not identical among all normal Japanese individuals (Table 2), but these individuals possessed the same set of associatively altered nucleotides at the *MDM2* polymorphic positions −628 in exon 1 and −466 in intron 1 (the nucleotides shown as italics in Table 2). We also found a C-to-T substitution in *$p14^{ARF}$* at position −4924 (Fig. 4) by sequencing the nucleotides upstream of the *$p14^{ARF}$* 4.5 kb promoters in normal individuals. Similarly, the −4924 polymorphism of *$p14^{ARF}$* was located in associative alterations in nucleotides (types α-γ in Table 3) with other *$p14^{ARF}$* polymorphisms at positions −2610 (an A-to-T substitution; Fig. A.4a−c) and −2221 to −2218 (an AA deletion; Fig. A.4d−f); the frequency of nucleotide types α−γ was 28.6 %, 57.1 %, and 14.3 %, respectively (Table 3).

Discussion. Gene nucleotide alterations and their frequency distributions have been analyzed in various populations, including different regions, ethnic groups, and patients with diseases; however, the nucleotide variations in normal individuals are not still understood sufficiently. Some *PTEN* polymorphisms have been observed in control populations [17, 2, 1, 19]. In Japanese or Chinese control subjects without diabetes or carcinomas, the frequency of the *PTEN* 5′-UTR −9 polymorphism (nucleotide G) is 2.5 % [2] and 6.5 % [19], respectively. This polymorphism is also detected in a Turkish population with glioblastoma multiforme (GBM) [25], but it is not found in a Danish Caucasian population, where some intron polymorphisms have been identified and association studies for diabetes have been conducted [26]. We found other *PTEN* 5′-UTR polymorphisms at positions −465 to −463 and position −404 in normal Japanese individuals (Figs 1−3), and we observed these polymorphisms in 1 (4.2 %) and 2 (8.3 %) alleles, respectively, of 24 alleles (Table 2). The frequency of these *PTEN* polymorphisms was approximately similar to the frequency of the *PTEN* 5′-UTR −9 polymorphism (2.5 %; [2]) in non-diabetic Japanese subjects

or the *PTEN* −1142 polymorphism in normal Japanese individuals (5.9%; [5]). Normal Japanese individuals, including individuals I and VIII with the *PTEN* 5′-UTR polymorphisms at positions −465 to −463 and −404, harbored an insertion in the *p53* promoter at the −824 to −818 poly(C) positions; substitutions in *MDM2* exon 1 and intron 1 at positions −628, −466, and −215 (SNP309) and in β_2- and β_3-*AR* exon 1 at codons 27 and 64; and a substitution or deletion in $p14^{ARF}$ at positions −4924, −2610, and −2221 to −2218 (Tables 2 and 3). The frequency of the *p53*, *MDM2*, and β_2- and β_3-*AR* polymorphisms was not markedly different from that previously observed in normal Japanese individuals [6] and control subjects who visited Japanese hospitals for a checkup [27, 12, 13, 15]. The −3735 polymorphism of $p14^{ARF}$ was not observed in our normal Japanese individuals (Table 2), and a Korean population had this polymorphism at a low frequency (0.03 %; [11]). The β_2-*AR* codon 27 and β_3-*AR* codon 64 polymorphisms are observed in various races, including Asians [13], Europeans [23, 28], and Caucasians [29]. The β_2-*AR* codon 27 polymorphism is observed at a higher frequency in Caucasian populations, but the frequency of the β_3-*AR* codon 64 polymorphism is similar to that observed in African-American and Hispanic-Latino populations [30]. Further, the *MDM2* SNP309 polymorphism is distributed differently between populations, including in patients and non-diseased patients [27], distinct regions [4], and ethnic groups [31, 18]. Some polymorphic nucleotides, including those of $p14^{ARF}$ at positions −4256, −3631 and −1477, are located in associatively altered nucleotides in a Korean population [11]. Normal Japanese individuals harbor simultaneous alterations, including those in the *PTEN* promoter at position −1124 and the $p15^{INK4b}$ (a cyclin-dependent kinase

Table 2

***PTEN*, *p53*, $p14^{ARF}$, *MDM2*, and β_2- and β_3-*AR* polymorphisms**

Individual number	*PTEN* 5′-UTR −465 to −463	*PTEN* 5′-UTR −404	*p53* Promoter −824 to −818	*MDM2* Exon 1 −628[1]	*MDM2* Intron 1 −466[1]	*MDM2* Intron 1 −215	β_2-*AR* Exon 1 codon 27	β_3-*AR* Exon 1 codon 64	$p14^{ARF}$ Promoter −3735
I	3G/2G	C/C	7C/8C	*A/G*	*C/T*	T/T	Gln/Gln	Trp/Arg	A/A
II	3G/3G	C/C	7C/8C	*A/A*	*C/C*	G/G	Gln/Gln	Trp/Trp	A/A
III	3G/3G	C/C	7C/8C	*A/A*	*C/C*	G/G	Gln/Glu	Trp/Trp	A/A
IV	3G/3G	C/C	7C/8C	*A/A*	*C/C*	T/T	Gln/Gln	Trp/Trp	A/A
V	3G/3G	C/C	7C/7C	*A/A*	*C/C*	T/G	Gln/Gln	Trp/Trp	A/A
VI	3G/3G	C/C	7C/8C	*A/G*	*C/T*	T/T	Gln/Gln	Trp/Trp	A/A
VII	3G/3G	C/C	7C/8C	*A/G*	*C/T*	T/T	Gln/Gln	Trp/Arg	A/A
VIII	3G/3G	T/T	7C/8C	*A/A*	*C/C*	G/G	Gln/Gln	Trp/Arg	A/A
IX	3G/3G	C/C	7C/7C	*A/G*	*C/T*	T/G	Gln/Gln	Trp/Trp	A/A
X	3G/3G	C/C	7C/7C	*G/G*	*T/T*	T/T	Gln/Gln	Trp/Trp	A/A
XI	3G/3G	C/C	8C/8C	*A/A*	*C/C*	G/G	Gln/Gln	Trp/Arg	A/A
XII	3G/3G	C/C	8C/8C	*A/A*	*C/C*	G/G	Gln/Gln	Trp/Trp	A/A

Indications. Nucleotide sequences were determined by direct sequencing of PCR products or by digesting PCR products with a restriction enzyme. The sequencing reactions were performed with the primers for *PTEN* (positions −375 to −398 and −247 to −270), *p53* (positions −669 to −690), $p14^{ARF}$ (positions −4050 to −4028), and *MDM2* (positions −725 to −704 and −310 to −289) (Table 1), and the digested products were separated and detected on agarose gels. 3G and 2G show *PTEN* nucleotides GGG and GG, respectively, at positions −465 to −463, and 7C and 8C show *p53* poly(C) nucleotides CCCCCCC and CCCCCCCC, respectively, at positions −824 to −818. β_2-*AR* Gln/Gln and β_3-*AR* Trp/Trp express homozygous amino acids glutamine at codon 27 and tryptophan at codon 64, respectively. β_2-*AR* Gln/Glu and β_3-*AR* Trp/Arg show heterozygous nucleotides consisting of glutamine and glutamic acid at codon 27 and of tryptophan and arginine at codon 64, respectively. [1]The characters in italics indicate nucleotides that are included in a set of associatively altered nucleotides.

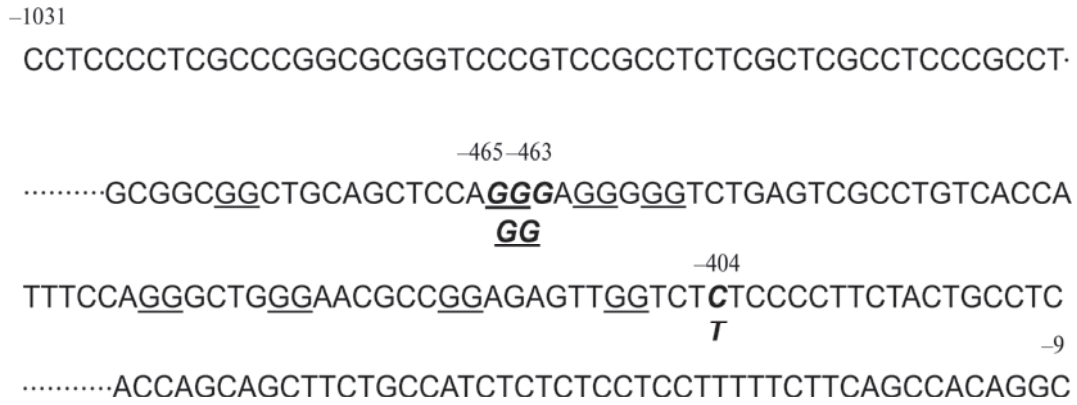

Fig. 1. Polymorphic nucleotides located in the *PTEN* 5′-UTR. Nucleotide alterations found in normal individuals are indicated by bold italics (***GGG*** or ***GG*** at positions −465 to −463 (a G deletion) and ***C*** or ***T*** at position −404 (a C-to-T substitution)) in the alignment of the *PTEN* 5′-UTR sequence deposited in GenBank (accession no. AF067844). The underlined GG dinucleotides show the distribution of the putative G-quadruplex sequences

(CDK) inhibitor) promoter at position −699 [5] or in the *p53* promoter at the poly(C) positions and the *MDM2* exon 1 and intron 2 nucleotides at positions −628 and −466 [6]. Correspondingly, most of the normal Japanese individuals examined in the present study had simultaneously polymorphisms at ≥2 positions in *PTEN*, *p53*, $p14^{ARF}$, *MDM2*, and β_2- and β_3-*AR* (Tables 2 and 3). In the present study, healthy Japanese individuals harbored polymorphisms in the *PTEN* 5′-UTR at positions −465 to −463 and −404 together with other polymorphisms in the promoter, intron, and exon regions of the other genes, and individual nucleotide alterations were distributed differently in the Japanese individuals. In addition, these individuals harbored some polymorphic nucleotides located in the same set of associatively altered nucleotides at positions, including positions −4929, −2610, and −2221 to −2218 of $p14^{ARF}$. Our results provide helpful information for studies investigating the distribution of polymorphisms in different populations, including those of regions, races, and patients, and normal individuals.

The identification of common polymorphism patterns in the human genome has been pursued by the research community [32]. In a Korean population, some polymorphic positions in the $p14^{ARF}$ promoter, including position −3631, are included in a haplotype block (a set of single-nucleotide polymorphism (SNP)alleles in a chromosomal region) [11]. Polymorphisms located in a haplotype block are useful for genotyping studies in populations with similar polymorphic alleles. The polymorphic nucleotides of *MDM2* at positions −628 and −466 observed in our Japanese samples were located in the same set of associative nucleotide alterations (Table 2), similar to those previously observed in normal Japanese individuals [6]. Likewise, the $p14^{ARF}$ −4924 polymorphism found in our samples was located in such a set of associatively altered nucleotides with other $p14^{ARF}$ polymorphisms

Table 3

$p14^{ARF}$ polymorphic nucleotides associatively altered in normal Japanese individuals

Polimorphic types	$p14^{ARF}$			Frequency, %
	−4924	−2610[1]	−2218 to −2221[1]	
Type α	C/C	A/A	4A/4A	28.6
Type β	C/T	A/T	4A/2A	57.1
Type γ	T/T	T/T	2A/2A	14.3

Indications. [1]Genomic DNA was amplified by PCR with forward primers at positions −2986 to −2965 and −2702 to −2682 and reverse primers at positions −2532 to −2553 and −2157 to −2178. The nucleotide sequences were determined by direct sequencing of the PCR products; the sequencing reactions were performed with the primers at positions −2702 to −2682 and −2300 to −2279. 4A and 2A show $p14^{ARF}$ polymorphic nucleotides AAAA and AA, respectively, at positions −2218 to −2221.

(Table 3). On the other hand, the *PTEN* polymorphisms at positions −465 to −463 and −404 were not involved in the set of associative alterations (Table 2). This result appears to contrast with previous results observed by the *PTEN* intronic polymorphisms in non-cancer patients and tumor patients [17]. Polymorphic nucleotides associatively altered in individuals may be distributed differently within gene regions. The strength of linkage disequilibrium of the *MDM2* SNP309 polymorphism with other *MDM2* polymorphisms differs between Ashkenazi Jewish, Caucasian, and African-American populations [18]. The polymorphisms, including the *PTEN* polymorphisms, found in the current study may be candidate polymorphisms for the construction of common polymorphism patterns in populations, including other ethnic groups and diseased patients. Further investigations with a larger number of samples are needed to determine the degree of linkage strength among polymorphisms in populations.

Gene transcript analysis with a reporter gene revealed that deletion of a region of the *PTEN* 5′-UTR sequence alters reporter gene expression in HEK293 cells [33]. The cellular senescence-inhibited gene (CSIG) binds to *PTEN* mRNA, and inhibition of CSIG gene expression by using small interfering RNA modifies the change in reporter expression following insertion of the *PTEN* 5′-UTR [33]. In Cos1 cells or Rat1 fibroblasts overexpressing insulin receptors, the PTEN protein level and the molecular response of Akt, a protein kinase, to treatment with insulin are differentially induced by the *PTEN* −9 polymorphism [2]. The alignment of G-quadruplex nucleotides binds to cellular proteins [34] and can regulate cellular gene expression [35–37]. A search for G-quadruplex motifs by using the GRSDB2 database ([38]; http://bioinformatics.ramapo.edu/GQRS/) showed that the *PTEN* polymorphisms are located within or around a G-quadruplex sequence (which is indicated by the underlined nucleotides GG in Fig. 1). A 5′-UTR polymorphism in *BRCA2* at position −26 reportedly alters the RNA secondary structure of the 5′-UTR and changes gene transcription activity in HeLa and MCF-7 cells [39]. The 5′-UTR nucleotides around the *PTEN* polymorphisms at positions −465 to −463 are partially included in the alignment of the 5′-GGGGAGGGGG-3′ sequence motif [40] or the 5′-GAGGAGGGGG-3′ motif [41] located in a gene regulatory region for platelet-derived growth factor A-chain [40] or tissue inhibitor of metalloproteinase 2 (*TIMP2*) [41]; the *TIMP2* sequence motif can interact with the Sp1 and Sp3 transcription factors [41]. We searched for transcription factors that can bind to sequences around the identified *PTEN* polymorphisms by using the TFSEARCH program (Searching Transcription Factor Binding Sites; http://mbs.cbrc.jp/research/db/TFSEARCH.html) with a matrix similarity of >0.7. We also predicted the RNA secondary structure of the *PTEN* 5′-UTR (from position −1031 to position −9; accession no. AF067844) with the CENTROIDFOLD program ([42]; http://www.ncrna.org/centroidfold/). TFSEARCH database analysis revealed that the *PTEN* polymorphisms affected the similarity of sequences of the substrates for putative transcription factors, for example, the similarity for MZF [43] at deleted nucleotide positions −465 to −463 and that for c-Rel [44] at substituted nucleotide position −404 in *PTEN*; similar results were also obtained by other polymorphisms (in a matrix similarity of >0.65), which affected, e.g., the similarity for TATA-binding protein at substituted or deleted positions −4924, −2610, and −2221 to −2218 in $p14^{ARF}$. Computational assessment with the CENTROIDFOLD program showed that the RNA structure was partially altered by the *PTEN* polymorphisms (Fig. A.5a–c). Similarly, the *PTEN* 5′-UTR −9 polymorphism changed the similarity for certain transcription factors (e.g., p300; [45]) and partially altered the structure of the 5′-UTR (Fig. A.5 a and d). These results indicate that the polymorphisms, including the *PTEN* polymorphisms, can change the binding ability of known transcription factors to the regulatory regions and that the *PTEN* polymorphisms can influence the interactions of complementary nucleotide sequences.

PTEN is differentially expressed in control subjects [1] and non-tumor tissues [8, 9], and *PTEN* gene expression has been reported to differ among patients, including those with Cowden syndrome, an autosomal dominant disorder [1], and in individual mononuclear cells from bone marrow or peripheral blood obtained from

patients with normal acute myeloid leukemia ([46]; data shown in accession no. GSE12417 in the NCBI Gene Expression Omnibus database). Gene expression analysis with the Cancer Genome Atlas ([47]; http://cancergenome.nih.gov/) has shown that *PTEN* levels differ among patients with cancer, including those with GBM; a number of patients with cancer harbor somatic mutations in the exonic regions of the gene; however, other patients show different gene expression levels in the absence of these mutations. The mechanisms underlying the variable expression of *PTEN* among normal individuals and patients with disease remain unclear. The *PTEN* nucleotide alterations at polymorphic positions change transcription factor binding and affect nucleotide-nucleotide interactions in silico, and *PTEN* mRNA interacts with cellular protein that can alter gene expression depending on the sequence of the *PTEN* 5′-UTR [33]. Our results provide possible clues for clarifying the molecular machinery underlying the gene expression observed in individuals, including patients.

The polymorphisms of *MDM2* at position SNP309, of β_2- and β_3-*AR* at codons 27 and 64, and of *p53* at codon 72 are associated with individual predisposition to diseases such as diabetes [12, 23, 48] and carcinomas [49, 27, 15, 16] in various populations, including the Japanese population. The frequency of the β_2-*AR* polymorphism Gln/Glu at codon 27 is increased in Caucasian females with obesity, but is decreased in obese males [29]. The β_3-*AR* polymorphism Trp/Arg at codon 64 is associated with cardiovascular risk factors in obese Hungarian children [28]. The $p14^{ARF}$ polymorphism at position −3631 has been associated with breast cancer in a British population [50], while the $p14^{ARF}$ polymorphism at −3735 is associated with diabetes in a French population with Europid ancestry [10]. The risk of chemical poisoning is associated with a polymorphism in intron 1 of $p14^{ARF}$ [51] and the risk of developing tumors is associated with a polymorphism in intron 4 of *PTEN* [19] in a Chinese population, and these associations have been shown to be dependent on the presence of another promoter polymorphism (Del1518) in the *MDM2* gene [51] and the absence of the 5′-UTR −9 polymorphism in the *PTEN* gene [19], respectively. The simultaneous presence of other polymorphisms has also been shown to alter the degree of disease risk by gene polymorphisms [15, 49, 39]. Normal Japanese individuals did not harbor the $p14^{ARF}$ −3735 polymorphism, but harbored polymorphisms in the *PTEN* 5′-UTR, in the promoter poly(C) nucleotides of *p53* and other promoter nucleotides of $p14^{ARF}$, and in intron 1 and exon 1 of *MDM2* and β_2- and β_3-*AR* (Tables 2 and 3). Individuals II, III, VI, VII, and IX–XII harbored some simultaneous alterations in *MDM2*, β_2- and β_3-*AR*, and *p53* (Table 2), while individuals IV and V harbored either the *p53* poly(C) polymorphism or the *MDM2* SNP309 polymorphism, respectively (Table 2). Methyl-cytosine-phospho-guanine(CpG)sequences induced by DNA methyltransferases interact with methyl-CpG binding proteins and modulate cellular gene expression [52]. The SNP309 polymorphism of *MDM2* is adjacent to a CpG sequence. Aditionally the cellular responses of β_2- or β_3-AR to an agonist for each receptor differ in individuals with the β_2-*AR* codon 27 polymorphism [53] or the β_3-*AR* codon 64 polymorphism [54]. Individuals I and VIII, with the *PTEN* polymorphisms, harbored simultaneously the *p53*, *MDM2*, and β_3-*AR* polymorphisms (Table 2). A C-to-T substitution (C609T) in *NQO1*, which was commonly observed in our individuals, has been associated with promoter methylation of the O^6-methylguanine-DNA methyltransferase and $p16^{INK4a}$ (another CDK inhibitor) genes [55]. In our individual samples, the nucleotide A in the $p16^{INK4a}$ promoter at position −191 was substituted for nucleotide G, which is located within the CpG sequence (Fig. A.6 c and d). The −191 polymorphism was located in some individuals with the *PTEN*, *MDM2*, *p53*, or β_3-*AR* polymorphism, and the polymorphic frequency of $p16^{INK4a}$ was similar to that of *NQO1* observed in our samples. Furthermore, the $p16^{INK4a}$ −191 polymorphism (Fig. A.6 c and d) and the $p14^{ARF}$ −4924 (Fig. 4 b and c), −3631 (Fig. A.6 a and b), −2610 (Fig. A.4 b and c), and −2218 to −2221 (Fig. A.4 e and f) polymorphisms were observed in normal individuals, including individual I harboring four polymorphisms and individual IV harboring one polymorphism (Table A.1 and some data not shown). In follow-up studies and population-based studies to determine the association of polymorphisms

to individual predispositions, polymorphisms are useful markers of an individual's constitution and as information for personalized medicine. Our findings provide useful information for studies on the determination of polymorphism patterns that exhibit an individual's predisposition, including disease, and contribute to a better understanding of individual nucleotide variations. Further studies are needed to clarify individual differences in nucleotide sequences.

In the present study, we have shown that polymorphisms are observed in healthy Japanese individuals, including those at positions −465 to −463 and −404 of *PTEN* and position −4924 of $p14^{ARF}$, and that these individuals have a different distribution of polymorphisms in the 5′-UTR, promoter, intron, and exon regions of *PTEN*, *p53*, $p14^{ARF}$, *MDM2*, β_2- and β_3-*AR* and also harbor some polymorphic nucleotides located in the same set of associative nucleotide alterations. Most of the individuals with a nucleotide alteration harbored other polymorphisms. Our results show that multiple nucleotides, including the *PTEN* nucleotides, are altered in healthy Japanese individuals and provide useful information for genotyping studies in individuals and populations.

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ПОЛИМОРФИЗМ 5′-UTR ГЕНА *PTEN* И ДРУГИЕ ГЕННЫЕ ПОЛИМОРФИЗМЫ СРЕДИ ЗДОРОВЫХ ЯПОНСКИХ ИНДИВИДОВ

Полиморфизмы широко распространены в популяциях, включая регионы, этнические группы, а также среди больных пациентов. Для исследования изменчивости нуклеотидных последовательностей у нормальных индивидов мы выделили геномную ДНК из крови здоровых японцев и секвенировали 5′-нетранслируемый участок (5′-UTR) гена фосфатазы и гомолога тензина (*PTEN*), промоторные последовательности, интроны и экзоны генов *p53* и $p14^{ARF}$, ингибитора супрессора опухолевого роста *p53* (*MDM2*) и генов β_2- и β_3-адренорецепторов (-*AR*). Мы обнаружили полиморфизмы в этих участках, включая делецию в положении с −465 до −463, замену в положениях −404 в гене *PTEN* и −4924 *вр* в гене 14^{ARF} у нормальных индивидов. Индивиды с полиморфизмом *PTEN* и без него имели различное распределение полиморфных вариантов в генах *p53*, *MDM2* и β_3-*AR*, а также содержали последовательность асоциированных полиморфных нуклеотидов. Наши результаты показывают наличие полиморфных нуклеотидов, включая полиморфизм в гене *PTEN*, среди здоровых японских индивидов, что обеспечивает перспективность исследований по генотипированию индивидов и популяционных исследований.

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