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Genetic Polymorphisms of the Human Thyroid Peroxidase Gene Using Amplified Fragment Length Polymorphism: Application to the Determination of Paternity in a Korean Population.

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Abstract: Genetic polymorphisms due to variation in the number of tandem repeats of DNA sequences(VNTRs) provides a useful means for discrimination between individuals. Allele and genotype frequencies of the highly polymorphic Human Thyroid Peroxidase(TPO) gene were determined in Korean population samples by using PCR followed by polyacrylamide gel electrophoresis, a procedure called the amplified fragment length polymorphism(Amp-FLP) technique. In 123 unrelated Korean individuals 10 different alleles and 29 genotypes were observed. The TPO gene demonstrated a heterozygosity of 0.707 and the power of exclusion(POE) was 0.945. The probability of having the same DNA band within two unrelated individuals was 14.6×10^{-2}. The distribution of observed genotypes conformed to Hardy-Weinberg equilibrium(x^2=4.48, 0.05<P<0.1, df=2). In 30 paternity cases Mendelian inheritance for the alleles at the TPO gene could be demonstrated in all cases, and the mutation rate was 0.075.

Key Words: TPO, VNTR, Polymorphism, Allele frequency, PCR, Amp-FLP.

INTRODUCTION

The class of polymorphic loci, known as variable number of tandem repeats or VNTRs (Variation Number of Tandem Repeat), are highly polymorphic in humans[17,35]. Some of the most polymorphic loci contain VNTR "core" units consisting of noncoding sequences of nucleotides[24]. A recent development in DNA technology, Amplified Fragment Length Polymorphism(Amp-FLP)[44] technique using Polymerase Chain Reaction (PCR)[16,40], provides a valuable tool for the detection of DNA polymorphism of VNTR markers. By means of appropriate DNA primers, which are complementary to sequences flanking the repeat region of the VNTR locus, it is possible to amplify the target sequence[2]. After amplification of the repetitive core sequences, the amplified DNA can be resolved using agarose gel or polyacrylamide gel electrophoresis. The different-sized DNA fragments representing alleles can then be visualized using ethidium bromide staining procedure[22]. Among previously reported VNTR genetic markers, the polymorphism of Thyroid Peroxidase(TPO)gene was determined by Amp-FLP technique. TPO is a membrane-bound glycosylated protein that plays a key role in the biosynthesis of thyroid hormones[36]. The gene for this enzyme spans over 150 kb on

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the short arm of chromosome 2 and consists of 17 exons and 16 introns[11], almost entirely sequenced[28]. Recently, the structure of a highly polymorphic region of the TPO gene, which was previously identified by using a TPO cDNA probe with EcoRI enzyme was described[5,6]. Polymorphism, which lies 1.9kb downstream of exon 10, is due to VNTRs, each 50 bp long[43]. In this study, the allele and genotype frequency distribution and heterozygosity of TPO gene were determined before TPO gene could be adopted for routine use. Mutation rates in paternity inclusion cases were also determined to examine the Mendelian inheritance of the polymorphism of TPO gene in Koreans.

MATERIALS AND METHODS

Proteinase K(20unit/mg) and *Taq* polymerase were obtained from Boehriner. Agarose(type 1) was purchased from Sigma. All other chemicals were analytical grade. 123 randomly selected healthy Korean individuals and 30 paternity inclusion family groups from Seoul Medical Science Institute were investigated.

Synthesis of Sequence Specific Primers

Primers were designed using the nucleic acid sequence of TPO gene. Amplification primers were synthesized using a 392 DNA/RNA synthesizer.

DNA extraction

Blood was collected into EDTA tubes and stored at 4℃. Aliquots were frozen and thawed. An equal volume of 15mM sodium citrate and 150mM sodium chloride(1XSSC) was added and the white cells were collected by centrifugation. The white cell pellet was resuspended in 1ml 1XSSC and centrifugation step was repeated. The pellet was suspended in 375ul of 0.2M sodium acetate(pH 7.0) 25ul of 10% SDS and 20ul proteinase K(10mg/ml) were added, the suspension was mixed thoroughly and then incubated at 56℃ for two hours. After two phenol/chloroform extractions DNA was recovered from the aqueous phase by the addition of two volumes of absolute ethanol. The DNA pellet was recovered by addition of 500ul of ethanol. After centrifugation, the DNA pellet was rinsed with 1ml of 80% ethanol and dried[19,30].

Amplification of DNA

Two unique oligonucleotide primers flanking the repeat sequence of TPO gene were used - TP1 5'-GTCAACCTCACGTTTGAGCG and TP2 5'-ATGCAGTGGGCTTCGAACTG (TPO sequence - GenBank Accession No. M 68651)[6]. Each PCR mixture(20ul) containing Taq 0.3U, 1uM of each primer, dNTPs (2mM) and $MgCl_2$ (2.5 mM) was subjected to 35 cycles for 1 min 30 sec at 94℃, 2 min at 68℃, 3 min at 72℃ by automated thermal cycler (GeneAmp PCR system 9600. Perkin Elmer Cetus Inc.). DNA without primer was used as a negative control. After addition of 2 ul loading buffer (30%(v/v) glycerol stained with bromophenol blue and xylene cyanol), the PCR products were loaded in 5 % polyacrylamide gel electrophoresis(PAGE) in a glass plate apparatus(Bio Rad Co. Ltd) for precise analysis of the amplified DNA products[15]. Gels were examined under UV illumination and documented by photography[34].

Statistical evaluation

Allele size was analyzed using the Gene Scanner(Applied Biosystems, Foster City, CA). The degree of individual specificity of genotypes determined by TPO gene can be estimated from the heterozygosity H. The mean allele frequency q at a locus is given by $(1-H)$, and assuming that all alleles share the same population frequency q, then the probability that two randomly selected unrelated individuals possessing the same genotype can be estimated as $q^2(2-q)4,37$. The frequency of each

allele in the population was calculated from the numbers of each genotype that were collected in the sample set. From the allele frequency data the expected number of genotype frequencies was calculated under the assumption of Hardy-Weinberg(H-W) expectations[10]. A x^2 (chi-square method; $x^2 = \Sigma$(observed number-expected number)^2/expected number) comparing observed and expected genotypes was also performed to assess whether the Korean population samples conforms to H-E expectations[9]. The power of exclusion(POE) was calculated as POE=1-$\Sigma(Pi)2$, where Pi represents the frequency of each genotype[14,29].

RESULTS

Allele and Genotype frequency in the amplified TPO gene

123 unrelated Korean individuals were able to be typed by TPO Amp-FLP assay. No false-positive amplifications were obtained in the 36 homozygous and 87 heterozygous individuals investigated. The amplified DNA products of TPO gene by Amp-FLP were determined from 5% polyacrylamide gels. Table 1 shows the allele frequencies of TPO locus in 123 unrelated Koreans. The observed number of genotypes as well as the expected number of genotypes based on the assumption of H-W expectations for Koreans(Table 2). In this study, a total of 10 different alleles and 29 genotypes were observed. Allele 15 (750bps; f=0.25) was most commonly observed in Koreans(Table 3). The other alleles occurred at frequencies of 0.04 - 0.17 and the amplified DNA fragments of TPO gene ranged between 300 and 800 bps in length. In TPO genotypes, allele 10/15(heterozygote; f=0.11) and allele 15(homozygote; f=0. 11) were frequently observed(Table 2). The Korean population sample was in agreement with expectations of H-W equilibrium for TPO gene (x^2=4.49, $0.05<P<0.1$, df=2)(Table 3). The observed and expected heterozygosity of TPO gene were 0.707 and 0.764 respectively (Table 4). POE(Power of Exclusion) value of TPO gene was 0.945 and probability of 2 unrelated individuals possessing the same genotypes was $14.6x10^{-2}$. Fig.1 shows frequency distribution of the TPO alleles and amplification products of highly polymorphic TPO alleles in a sample of unrelated Koreans were shown in Fig.2.

Mutation rate

Prior to applying to the analysis of family relationships, the mutation rate of these polymorphic loci must be determined. We have studied the mutation rate in 30 paternity inclusion families(Table 4). The observed mutation rate was 0.075(Table 4a). The frequency of mutant bands among 30 cases(40 offsprings) of paternity inclusion was 0.10(Table 4b). The 3 mutations detected on the TPO gene were distributed between those of paternal and maternal origin(Table 4c).

Application to paternity

In our family study a Mendelian pattern of inheritance was observed in 30 paternity cases where the paternity was confirmed with previous experiments[32,33]. The TPO genetic poly morphism study on 30 paternity cases using by Amp-FLP correlated well with the RFLP analysis by multilocus and single locus probes procedures reported previously[32,33]. Examples of paternity analysis were shown in Fig. 3. Family case C (Fig.3) was the case of paternity exclusion, where none of the non-maternal bands in the child matched those of the alleged father.

DISCUSSION

Recent studies on the human genome have revealed an increasing number of DNA segments that display a high degree of polymorphism[2]. Several characteristics of VNTRs - the large number of alleles, their high het-

Table 1. TPO allele frequencies in 123 unrelated Koreans using AMP-FLP

TPO alleles (Repeats)	Size of DNA fragment (bps)	Number observed	Frequency observed
6	300	10	0.04
7	350	29	0.12
8	400	5	0.02
9	450	42	0.17
10	500	42	0.17
12	600	13	0.05
13	650	10	0.04
14	700	25	0.10
15	750	62	0.25
16	800	8	0.03

erozygosities, and their frequent occurrence in the genome - make them highly informative for purposes of human gene mapping, identification of individuals, and determining the relationship between individuals[41]. Until recently RFLP(Restriction Fragment Length Polymorphism)analysis and Southern hybridization has been a common method identifying these DNA polymorphisms[32,33]. The RFLP analysis, however, is time-consuming and labor intensive and requires intact high-molecular weight DNA[24]. Analysis of VNTR loci by PCR amplification, referred to as Amp-FLP[16,40], can overcome some of the limitations of RFLP analysis[29]. The analysis of relatively small-sized VNTR loci by PCR followed by electrophoretic separation of the amplified DNA fragments, offers two obvious advantages over the classical RFLP analysis via Southern blot. Firstly, PCR enables the rapid analysis of samples containing very small amounts and even degraded DNA. Secondly, Amp-FLP analysis permits the resolution of the different alleles associated with the VNTR locus under investigation into discrete entities[1]. In this experiment, population genetic data in Koreans were determined, including allele frequency distribution, heterozygosity, probability of identity and mutation rate, for the purpose

Table 2. Observed and expected TPO genotypes in 123 unrelated Koreans using AMP-FLP

TPO Genotype	Number/Frequency obs. obs.	Number/Frequency exp. exp.
6	4/0.03	3.3/0.03
6/7	2/0.02	1.9/0.02
7	5/0.04	4.8/0.04
7/9	3/0.02	2.6/0.03
7/10	8/0.07	7.5/0.06
7/13	3/0.02	1.9/0.01
7/15	2/0.02	2.2/0.02
7/16	1/0.01	10/0.00
8	1/0.01	1.0/0.01
9	9/0.07	7.9/0.06
8/12	3/0.02	3.1/0.02
9/10	4/0.03	3.6/0.03
9/12	3/0.02	3.5/0.03
9/13	2/0.02	1.6/0.01
9/14	5/0.04	5.1/0.04
9/15	7/0.06	6.9/0.06
10	2/0.02	2.4/0.02
10/12	3/0.02	2.4/0.02
10/13	3/0.02	2.7/0.02
10/14	6/0.05	5.5/0.04
10/15	14/0.11	13.8/0.11
12/15	2/0.02	2.1/0.02
12/16	2/0.02	1.5/0.01
13/14	2/0.02	1.8/0.01
14	2/0.02	1.5/0.01
14/15	7/0.06	5.6/0.05
14/15	7/0.06	5.6/0.05
14/16	1/0.01	0.8/0.01
15	13/0.11	12.9/0.10
15/16	4/0.03	2.9/0.02

of using TPO gene in routine clinical work. The nomenclature for the alleles at the TPO gene has not been standardized. We have followed the allele designation suggested by Bikker et al.(1992). The size fragments of the TPO alleles permit resolution of individual alleles that differ in length by only one repeat unit consists of 50 bps[6]. This has made it possible to adopt a system of nomenclature that is based on the number of repeat units[43]. The alleles are labeled 6-16 from the smallest to the largest. The allele numbers indicate the number of repeat units inferred for each allele. The analysis of the TPO gene by using the Amp-

Table 3. Comparison of observed and expected heterozygosity of TPO gene in 123 unrelated Koreans

Hom_{obs}/Hom_{exp}	Het_{obs}/Het_{exp}	X^{2a}	Probability[b]	POE[c]
0.293/0.236	0.707/0.764	4.49 (0.05<P<0.1 df=2)	14.6×10^{-2}	0.945

a. chi-square method
$x^2=\Sigma$(observed number-expected number)^2/expected number
b. Probability, 2 unrelated individuals possessing the same genotypes
$P=q^2(2-q)$, q=the mean allele frequency
c. POE, Power of Exclusion
$POE=1-\Sigma(Pi)^2$, Pi represents the frequency of each genotype.

Table 4. Incidence of mutant bands in offspring in the 30 cases of paternity inclusion (40 offsprings)

A. Observed mutation rate			
No. of mutant alleles observed	Mutation rate /gamete		
3/40	0.075		
B. Frequency of mutant band			
No. of mutant bands in 40 off springs	No. of cases	% of cases	
0	27	90.0%	
1	3	10.0%	
C. Parental origin of mutant band			
	Maternal	Paternal	Total
No. of mutant bands	1	2	3

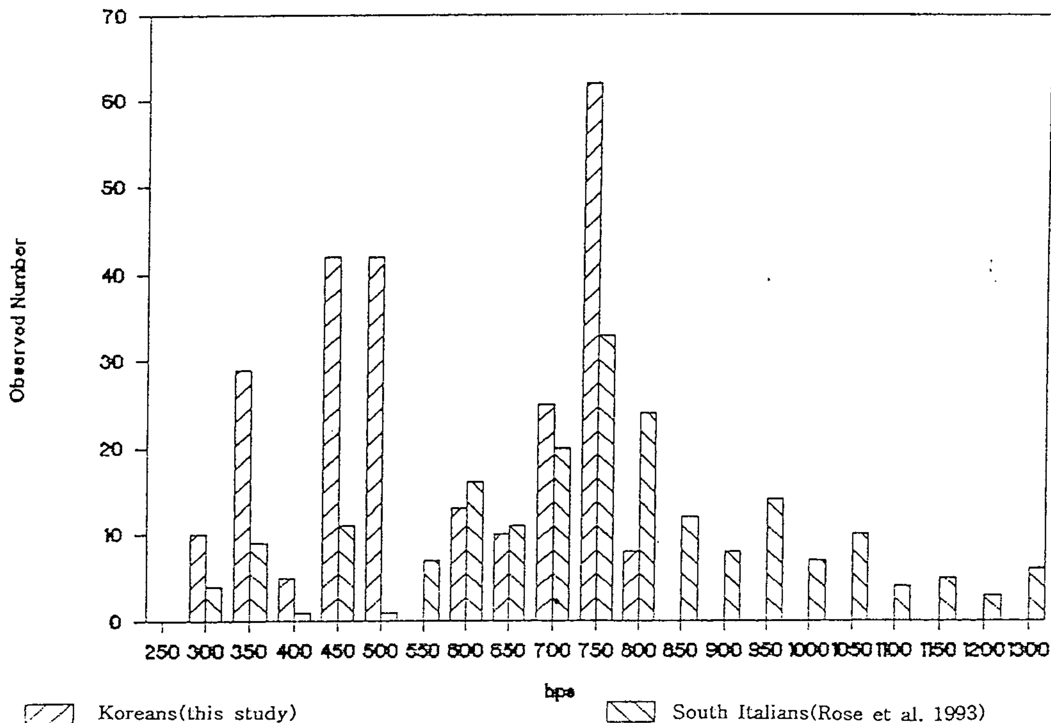

Fig. 1. Frequency distribution of the TPO alleles in a sample of 123 unrelated Koreans by Amp-FLP.

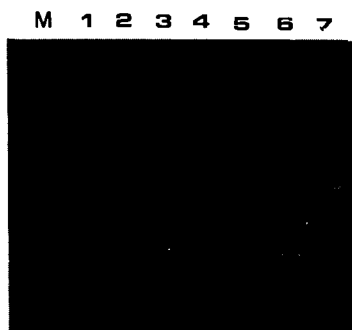

Fig. 2. Amplification products of TPO alleles in related individuals. Lanes 1-7 show patterns of genotypes. M(Molecular Weight Marker (PhiiX174 DNA/Hinfl).

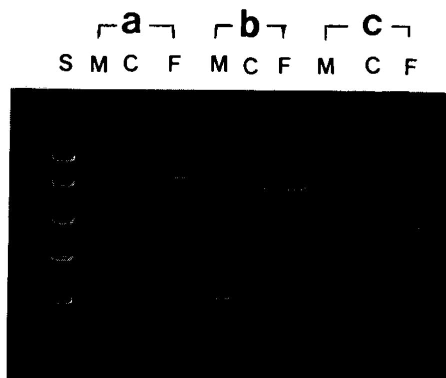

Fig. 3. Use of TPO gene in paternity testing, Case a, b: Paternity inclusion, Case c : Paternity exclusion, M: mother, C: child, F: father, S: Molecular Weight Marker, (PhiX174 DNA/Hinfl)

FLP technique has already been studied in 104 unrelated South Italian individuals[43]. 750 bps DNA fragment(15 repeats) showed the most frequent in both of the two data. In the number of TPO alleles, however, a definitely different data was shown between two ethnic groups. 10 different alleles(6-16 repeats)were found in Koreans, whereas 21 differnt alleles(6-26 repeats)were observed in South Italians[43] (Fig.1). It seems to suggest two possibilites. Firstly, it may have the technical problems, including extracted DNA, condition of PCR, PAGE and interpretation of its results. Secondly it might be due to the ethnic differences between two population groups. TPO Amp-FLP analysis has been used to establish paternity in 30 Korean families where paternity was confirmed with RFLP hybridization analysis using multilocus probes as well as single locus VNTR probes[32,33]. The mutation rate was very low(0.075, Table 1) and all of the results on 30 paternity cases were consistent with the previous data[32,33]. Furthermore, our population sample appears to meet H-W expectations for the TPO gene(x^2=4.48, $0.05<P<0.1$, *df*=2). The observed heterozygosity of TPO gene was 0.707. The probability and POE were 14.6×10^{-2} and 0.9451, respectively. From the above results, these TPO gene can be applied to the individual identification in forensic medicine[9] as well as determination of paternity[3,6,20] among Koreans. However, in order to obtain a high degree of individualization, additional genetic markers - such as the hypervariable region close to the 3' end of the apolipoprotein B gene[7,23], D1S80[8,12,25], D17S 30[22](also dsignated D17S5[13,39], ACTP gene[38], COL2A1 gene[21,26], D1S8[27], D4S43[18], and short tandem repeats(STRs)loci[31,42] could provide a powerful new tool for the DNA characterization for the identity[44]. In conclusion, the data on allele frequencies and mutation rates of TPO gene presented in this study provide a foundation for the use of the panel of hypervariable TPO gene in a Korean population.

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= 국문초록 =

한국인에서 중합효소연쇄반응을 이용한 Human Thyroid Peroxidase 유전자의 유전적 다형성에 관한 연구

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최근까지 개인식별검사는 주로 단백질의 다형성을 보는 것이었으나, 분자생물학의 발달로 개인식별 분야에도 DNA를 이용한 분석이 가능하게 되었다. 본 실험에서는 Thyroid Peroxidase(TPO)유전자의 다형성을 개인식별 및 친자감별검사에 이용하기 위하여 그 기초조사로 한국인에서 TPO유전자의 대립유전자 발현빈도와 돌연변이율 등을 포함한 평가실험을 실시하였다. 실험대상으로는 혈연관계가 없는 123명을 대상으로 말초혈액에서 DNA를 추출하고 PCR(중합효소연쇄반응)을 이용한 Amp-FLP방법을 이용하여 TPO 유전자를 증폭하였으며, polyacrylamide gel로 확인하였다. 그 결과 10개의 대립유전자와 29개의 유전형이 구분되었고, 이형접합성(Heterozygosity)은 70.7%였으며 돌연변이율은 0.075였고, 혈연 관계가 전혀 없는 두 사람이 동일한 유전자형을 가질 가능성(Probability)은 $14.6x10^{-2}$이었다. 또한 x^2=4.48, 0.05<P<1로써 Hardy-Weinberg법칙이 성립함을 보여주었다. 이상의 결과로 볼 때 TPO유전자는 한국인에서 개인식별이나 친자감별검사를 위한 유전적 표지자로 사용될 수 있을 것으로 사료된다.

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