III. 研究成果の刊行に関する一覧表

書籍

なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
副島美貴子 神田芳郎	TaqMan-based real-time PCR for genotyping common polymorphisms of haptoglobin (*HP*^{1} and *HP*^{2}).	Clinical Chemistry	54 (11)	1908-1913	2008
中村仁美 副島美貴子 Munkhtulga L 岩本禎彦 神田芳郎	Haptoglobin polymorphism in Mongolian population: comparison of the two genotyping methods.	Clin Chim Acta.	408 (1-2)	110-113	2009
副島美貴子 土屋雄二 江頭弘一 川野洋之 佐川公矯 神田芳郎	Development and validation of a SYBR Green I-based real-time polymerase chain reaction method for detection of haptoglobin gene deletion in clinical materials.	Transfusion	50 (6)	1322-1327	2010
副島美貴子 江頭弘一 川野洋之 川口　淳 佐川公矯 神田芳郎	Rapid Detection of Haptoglobin-Gene Deletion in Alkaline-Denatured Blood by Loop-Mediated Isothermal Amplification Reaction.	J Mol Diagn.	13 (3)	334-339	2011

神田芳郎 副島美貴子 川野洋之 江頭弘一 佐川公矯	輸血副作用原因遺伝子ハプトグロビン欠失アリルの輸血前診断法の検討	日本輸血細胞治療学会誌	57 (1)	34-38	2011

ＩＶ．研究成果の刊行物・別刷

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TaqMan-Based Real-Time PCR for Genotyping Common Polymorphisms of Haptoglobin (*HP*^{1} and *HP*^{2})

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BACKGROUND: The haptoglobin gene (*HP*) has 2 common codominant alleles (*HP*^{1} and *HP*^{2}) that account for 3 phenotypes. *HP*^{2} is generated by a 1.7-kb intragenic duplication of *HP*^{1}.

METHODS: We used the real-time TaqMan PCR system to develop an effective method for *HP* genotyping that allows us to evaluate the relative number of copies of the *HP*^{2} allele–specific junctional region of the 1.7-kb gene duplication (HP2) by comparing the intensity of the amplification signals to those of the *HP* promoter region (HP5′), which was used as the internal control. The difference in threshold cycles (ΔCt) between HP2 and HP5′ was used to assess *HP*^{2} copy number. In addition, the assay detects the *HP* deletion (*HP*^{del}) at the same time.

RESULTS: The mean $2^{-\Delta\Delta Ct}$ values (the HP2/HP5′ ratio) obtained from 123 samples of known *HP* genotypes clearly differentiated 2 nonoverlapping intervals that correspond to the *HP* genotypes. Ratios for *HP*^{2}/*HP*^{1} samples ranged from 0.34–0.50, *HP*^{2}/*HP*^{2} samples ranged from 0.79–0.98, and the absence of an *HP*^{2} allele signal was defined as *HP*^{1}/*HP*^{1}. We simultaneously detected *HP*^{del}. The assay produces results in <1 h.

CONCLUSIONS: The TaqMan-based real-time PCR method was successfully applied to *HP* genotyping. The method is easy to use in a molecular diagnosis laboratory, and its robustness and rapidity make it suitable for high-throughput analysis of large populations.

Haptoglobin (HP)[2] is a glycoprotein found in the plasma of all vertebrates. It binds hemoglobin to prevent both iron loss and kidney damage during hemolysis. Humans have a polymorphism in the gene encoding the protein because of 2 codominant *HP* (haptoglobin) alleles, *HP*^{1} and *HP*^{2}, that give rise to the 3 common HP phenotypes, HP1, HP2-1, and HP2 (*1*, *2*). The *HP*^{2} allele appears to have been generated by a 1.7-kb intragenic duplication of exons 3 and 4 of the *HP*^{1} allele. The frequencies of the *HP*^{1} and *HP*^{2} genotypes vary worldwide depending on the ethnic group, with the *HP*^{1} frequency varying from about 0.07 in parts of India to >0.7 in parts of West Africa and South America (*3*).

Because of its polymorphic nature, *HP* has been used as a genetic marker for identifying parentage and individuals, and several variant phenotypes have been reported, one of which, HP Johnson, occurs at low frequency in various populations (*3*). The molecular weight of the α chain is larger (23 kDa) than that encoded by *HP*^{2}, and Southern blotting has shown 3 tandem repeats of the same 1.7-kb DNA segment found in the *HP*^{2} gene duplication (*4*). Another variant, HP2-1 modified (HP2-1M), which exhibits greater HP1 protein production than HP2 because of a mutation in one of the interleukin-6 response elements in the promoter of the *HP*^{2} allele, occurs at relatively high frequencies in African populations (*5–7*). In addition, *HP*^{del}, which lacks an approximately 28-kb segment of chromosome 16 that extends from the *HP* promoter region to exon 5 of the haptoglobin-related gene, has been identified (*8*, *9*). This allele has been found only in East and Southeast Asian populations (*9–13*).

Both *HP*^{1} and *HP*^{2} alleles have been associated with susceptibility to diabetic, cardiovascular, inflammatory, and autoimmune diseases (*2*). Such associations may be explained by functional differences between the subtypes in the binding of hemoglobin and its rate of clearance from the plasma. Recent studies have identified profound differences between the antioxidant and immunomodulatory properties of

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[2] Nonstandard abbreviations: HP, haptoglobin; HP2–1M, HP2–1 modified; Ct, threshold cycle; HP2, region encompassing the breakpoint of the partial gene duplication in intron 4 of the *HP*^{2} allele; HP5′, 5′ region of *HP* exon 1; HP^{del}, region encompassing the *HP*^{del} breakpoint.

the HP1 and HP2 polypeptides *(14)*. There are also corresponding reports of negative associations with disease *(2)*. Thus, development of a simple, high-throughput *HP* genotyping method is needed to facilitate these association studies.

Several methods for phenotyping HP1 and HP2 have been described. High-pressure gel-permeation chromatography and gel electrophoresis methods rely on differences in the molecular sizes of HP1, HP2-1, and HP2 for typing *(14, 15)*. More recently, a single-chain antibody-based ELISA test was also established *(16)*. In addition, several *HP*-genotyping methods based on conventional PCR have been developed; however, these PCR-based methods need to amplify a relatively large DNA fragment to detect the 1.7-kb duplicated regions, are time-consuming, and require laborious post-PCR processing steps *(8, 17, 18)*. On the other hand, real-time PCR is a high-throughput, rapid, and sensitive method. It also eliminates post-PCR processing of PCR products, reducing the chances of carryover contamination. TaqMan assays (Applied Biosystems) use both amplification with gene-specific primers and fluorescence detection of target-specific probes. This method allows evaluation of RNA production or DNA genotyping of not only single-nucleotide polymorphisms but also gene dosage *(19–21)*.

We recently developed a TaqMan-based real-time PCR method that allows differentiation of *HP* and HP^{del} alleles by detecting the junctional region of the gene deletion and the promoter region of a gene that is deleted in HP^{del} *(22)*. To detect the junctional region of the 1.7-kb intragenic duplication of HP^2, we added the HP2 probe and primers in the TaqMan-based real-time PCR method mentioned above. This new method allows differentiation of HP^1 and HP^2 alleles by evaluation of the relative numbers of copies at the junctional region of the 1.7-kb intragenic duplication in HP^2 via comparison with the intensity of the amplification signals from an internal control (the promoter region of this gene).

Materials and Methods

The ethics committee of Kurume University School of Medicine approved this study. We used genomic DNA from 123 randomly selected Ghanaians from Accra and 7 Japanese individuals whose HP phenotypes and genotypes had previously been examined *(10)*.

We performed real-time PCR to detect 3 regions: (*a*) the region encompassing the breakpoint of the partial gene duplication in intron 4 of the HP^2 allele (HP2), (*b*) the 5′ region of *HP* exon 1 as an internal control to compare the intensity of the amplification signals (HP5′), and (*c*) the region encompassing the HP^{del} breakpoint (HP^{del}) to detect the HP^{del} allele at the same time. The 20-μL PCR reaction contained 6.1 pg to 100 ng genomic DNA, 10 μL of *Premix Ex Taq*™ (Perfect Real Time) (Takara Bio), and the following primers and TaqMan probes at the indicated concentrations: HP2-F and -R primers (300 nmol/L), HP2 probe (83 nmol/L) for the detection of HP^2, HP5′-F and -R primers (150 nmol/L), HP5′ probe (42 nmol/L) for the detection of the *HP* 5′ region, HP^{del}-F and -R primers (300 nmol/L), and HP^{del} probe (83 nmol/L) for the detection of HP^{del}. The PCR temperature profile was 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 30 s. The positions and sequences of the HP2-F and -R primers and the HP2 probe are as follows (GenBank no. M10935): HP2-F (5′-GGAGCTGCT CTGCACATCAA-3′, the reverse of the sequence of base pairs 4547–4566); HP2-R (5′-CCCTTTCAATGA ATTTCAGGGA-3′, sequence from base pairs 4448–4469); and HP2 probe (5′-CAL Fluor Orange 560-ACCCCGAATAGAAGCTCGCGAACTGTA-BHQ1-3′, the reverse of the sequence of base pairs 4511–4537). The positions and sequences of the HP5′-F and -R primers, the HP5′ probe, the HP^{del}-F and -R primers, and the HP^{del} probe have previously been described *(22)*. All oligonucleotides were synthesized by Biosearch Technologies. The relative positions of the HP2 and HP5′ probes in the HP^1 and HP^2 alleles are shown in Fig. 1. To increase the fluorescence signals, we carried out the PCR and signal detection in Sorenson ultraAmp PCR semiskirted 96-well white plates (Nippon Genetics). To monitor the progress of amplification, we measured the fluorescence at the end of each cycle with an Mx3000P system (Stratagene) equipped with excitation/emission filters of 492/516 nm (FAM), 535/555 nm (CAL Fluor Orange 560), and 585/610 nm (CAL Fluor Red 610). Data were collected and analyzed with MxPro™ software (version 4.00; Stratagene).

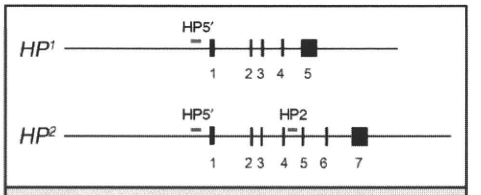

Fig. 1. Structures of the HP^1 and HP^2 alleles and relative positions of the HP2 and HP5′ probes.

The change in threshold cycle (ΔCt) of each sample was calculated as the Ct value of HP5′ (control) minus the Ct value of HP2 (target). The ΔΔCt of each sample was obtained by subtracting the ΔCt value of the sample from the ΔCt value of the reference. The

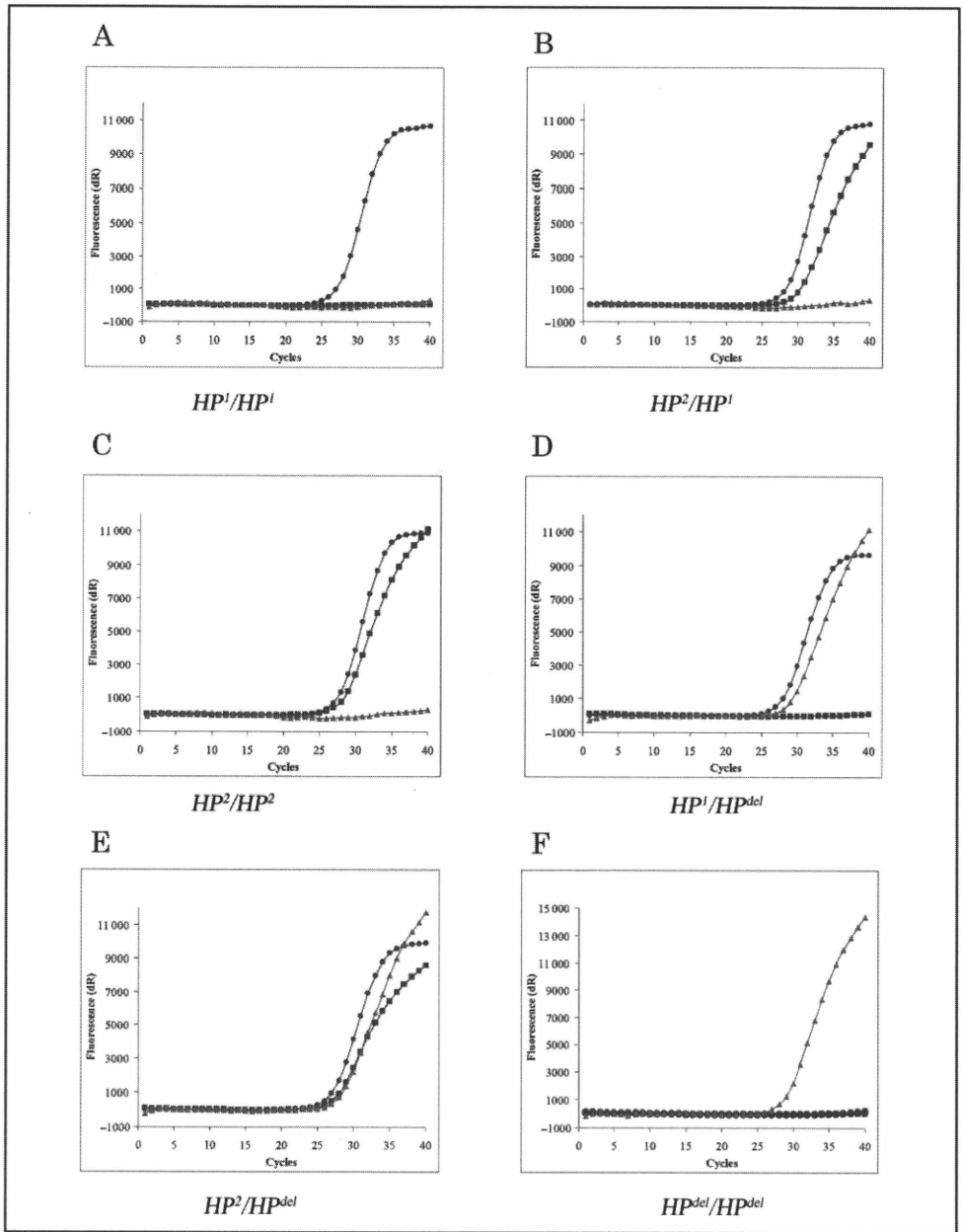

Fig. 2. Real-time amplification plots for HP2, HP5′, and HP^{del} with genomic DNA targets of various genotypes: HP^1/HP^1 (A), HP^2/HP^1 (B), HP^2/HP^2 (C), HP^1/HP^{del} (D), HP^2/HP^{del} (E), and HP^{del}/HP^{del} (F).

Red, blue, and green curves represent HP2, HP5′, and HP^{del}, respectively. dR, baseline-corrected raw fluorescence.

ΔCt reference value was obtained with 5 ng genomic DNA from an HP^2/HP^2 individual. The HP2/HP5′ ratio of each sample was calculated as $2^{-\Delta\Delta Ct\ sample}$.

Results

TRIPLEX PCR FOR HP2, HP5′, AND HP^{del}

The region chosen as the target sequence is located in the intronic sequence 5′ of the junction region of the gene duplication of the HP^2 allele (intron 4; Fig. 1). The HapMap and NCBI databases have not reported any single-nucleotide polymorphisms within this region. Although we could have used any of several genomic regions as an internal control for relative quantification of the HP^2-specific copy number, we selected the *HP* 5′ region as an internal control for 2 reasons: (*a*) We previously used the *HP* 5′ region to distinguish the HP^{del} allele (*22*), and (*b*) we might miscategorize individuals with HP^{del}, particularly HP^2/HP^{del} individuals as HP^2/HP^1, if we selected another region as the internal control. Thus, we used triplex PCR to detect the relative numbers of copies of the HP^2 allele and the HP^{del} allele by adding the HP2-F primer, HP2-R primer, and HP2 probe in the previously described TaqMan-based method, although the duplex PCR (using the HP5′ and HP2 probes) seems to be adequate for *HP* genotyping in many populations other than those of East and Southeast Asia. In this assay system, we observed amplification of the *HP* 5′ region (CAL Fluor Red 610 signal) without amplification of the HP^2 allele–specific region (CAL Fluor Orange 560 signal) or the HP^{del} allele–specific region (FAM signal) in the HP^1/HP^1 DNA sample (Fig. 2A), whereas we observed amplification of the HP^2 allele–specific region and the *HP* 5′ region without amplification of the HP^{del} allele–specific region in the HP^2/HP^1 and HP^2/HP^2 DNA samples (Fig. 2, B and C). We observed amplification of the *HP* 5′ region and the HP^{del} allele–specific region without amplification of the HP^2 allele–specific region in the HP^1/HP^{del} DNA sample (Fig. 2D) and obtained all 3 signals in the HP^2/HP^{del} DNA sample (Fig. 2E). Only the FAM signal was detected in the HP^{del}/HP^{del} DNA samples (Fig. 2F).

PCR EFFICIENCIES FOR HP2 AND HP5′

To determine the common *HP* genotypes, we then performed quantitative real-time PCR assays with TaqMan probes to evaluate the relative numbers of copies of the HP^2-specific sequence. We evaluated the effect of DNA quantity on PCR efficiency in a dilution series of genomic DNA (100, 25, 6.25, 1.56, 0.39, 0.098, 0.024, and 0.0061 ng/reaction) from HP^2/HP^1 or HP^2/HP^2 individuals. The calculated efficiency rates of the PCR for the HP^2-specific sequence were 108.9% and 102.3% for templates from HP^2/HP^1 and HP^2/HP^2 individuals, respectively, and those for HP5′ were 107.0% and 102.2% for templates from HP^2/HP^1 and HP^2/HP^2 individuals, respectively (Fig. 3). Because the efficiencies for both regions were very similar, the estimates of relative copy number obtained with these 2 Ct values were considered valid. In addition, the PCR reaction required <1 h. Thus, this method permits evaluation for both the common *HP* genotypes and the HP^{del} allele. This triplex PCR system is particularly suitable for East and South Asian populations, where the HP^{del} is observed.

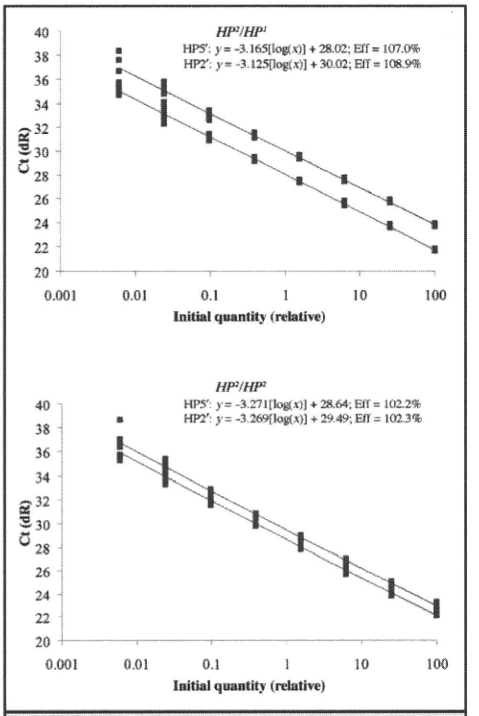

Fig. 3. HP2 and HP5′ calibration curves calculated from Ct data for 6.1 pg to 100 ng DNA template from HP^2/HP^1 and HP^2/HP^2 individuals.

Red and blue lines represent calculations for means of 6 data points for HP5′ (CAL Fluor Red 610) and HP2 (CAL Fluor Orange 560), respectively. Eff, efficiency; dR, baseline-corrected raw fluorescence.

VALIDATION OF THE ASSAY

To determine the quantity of genomic DNA necessary to obtain correct results, we calculated the results obtained with serially diluted samples of genomic DNA

Table 1. *HP* genotyping of 123 Ghanaian samples with a TaqMan assay.

HP genotype	No. of Individuals	Plate no.	HP2/HP5′ ratio in each assay[a]	Range	Total mean HP2/HP5′ ratio
HP^1/HP^1	21	1	0 (0)	0–0	0 (0)[b]
		2	0 (0)	0–0	
		3	0 (0)	0–0	
	14	4	0 (0)	0–0	
		5	0 (0)	0–0	
		6	0 (0)	0–0	
HP^2/HP^1	26	1	0.44 (0.03)	0.35–0.53	0.44 (0.036), 0.34–0.50[c]
		2	0.48 (0.03)	0.37–0.53	
		3	0.43 (0.03)	0.30–0.48	
	31	4	0.47 (0.03)	0.41–0.56	
		5	0.45 (0.03)	0.39–0.52	
		6	0.43 (0.03)	0.37–0.52	
HP^2/HP^2	15	1	0.87 (0.07)	0.74–0.99	0.88 (0.071), 0.79–0.98[c]
		2	0.88 (0.10)	0.69–1.10	
		3	0.92 (0.06)	0.83–1.10	
	16	4	0.89 (0.05)	0.81–0.97	
		5	0.89 (0.05)	0.80–0.99	
		6	0.82 (0.06)	0.72–0.94	

[a] Data are presented as the mean (SD).
[b] The range of HP5′ Ct values was 26.6–29.3; no HP2 Ct values were obtained.
[c] Data are presented as the mean (SD), range. Range data are the mean of 3 independent experiments.

from HP^2/HP^2 or HP^2/HP^i individuals as PCR templates. Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue11 presents mean (SD) Ct values for 0.0061–100 ng (2–33 000 copies of the control region) of genomic DNA from HP^2/HP^2 and HP^2/HP^i samples (n = 6). The ranges of ΔCt values (the HP5′ Ct value minus the HP2 Ct value) were −0.79 to −0.91 for HP^2/HP^2 and −1.85 to −2.07 for HP^2/HP^i. As expected, the variances of Ct values are higher with lower amounts of genomic DNA (0.0061 and 0.024 ng, i.e., 2–8 copies); however, mean ΔCt values did not differ appreciably at genomic DNA amounts >0.0061 ng. We observed no significant differences in Ct values between samples of the same genotype at genomic DNA amounts between 0.1–100 ng. Thus, the quantity of template DNA was not critical in this method. To obtain reference ΔCt values, we performed real-time PCR with 5 ng genomic DNA from an HP^2/HP^2 individual and obtained reference ΔCt values of −0.73 to −0.86 (data not shown).

We then examined 123 DNA samples from Ghanaians whose *HP* phenotypes and genotypes had previously been determined (*10*). HP5′ Ct values ranged from 26.6–29.3, and we obtained no HP2 Ct values for the 35 HP^i/HP^i individuals (n = 105). This absence of an HP^2 signal was defined as indicating an HP^i/HP^i genotype. Mean (SD) ΔCt values for 57 HP^2/HP^1 individuals were −1.99 (0.11) (range, −1.64 to −2.52; n = 171), and those for 31 HP^2/HP^2 individuals were −1.00 (0.11) (range, −0.62 to −1.29; n = 93) (data not shown). We then calculated the HP2/HP5′ ratio with the ΔΔCt value of each sample. Table 1 shows the average of 6 independent assays (3 independent assays of each sample). In each assay, the quantitative results obtained with the present real-time PCR assay were concordant with those obtained with previous phenotype and genotype assays. We observed no overlap between the ranges of values that corresponded to the HP^2/HP^2 and HP^2/HP^i genotypes. The mean (SD) HP2/HP5′ ratios were 0.44 (0.036) for 57 HP^2/HP^1 individuals and 0.88 (0.071) for 31 HP^2/HP^2 individuals.

Although we have examined only a single individual for each of the $HP^{\Delta ei}$ genotypes (i.e., $HP^i/HP^{\Delta ei}$, $HP^2/HP^{\Delta ei}$, and $HP^{\Delta ei}/HP^{\Delta ei}$), the results of 12 replicate assays were identical, suggesting that our application of the present method to genotyping of the common *HP* alleles and to detecting $HP^{\Delta ei}$ was successful.

Discussion

We have developed an effective method for genotyping common HP polymorphisms with the real-time TaqMan PCR system; however, the main limitation of this method, compared with Southern blotting, phenotyping, and conventional PCR methods, is misidentification of rare variants such as HP Johnson and HP2-1M (*4*). The frequency of the HP Johnson allele is very low in many populations (*3*), whereas the incidence of HP2-1M can be as high as 10% (in Africans), which is not negligible (*6*, *7*). In fact, we have 15 samples from individuals with the HP2-1M phenotype but without HP Johnson in the sample of 123 Ghanaians. Because the present method calculated only the copy number of the HP^2-specific sequence relative to that of the internal control sequence, we identified HP2-1M as HP^2/HP^1 in the present real time PCR method. In addition, we may score an HP Johnson allele as HP^2/HP^2. Overcoming this miscategorization seems to be difficult with the present assay system. On the other hand, if instead of the HP^{del} probe we were to use a probe in the assay system that includes the substitution of the causal base at the promoter that is responsible for the HP2-1M phenotype (A-61C), we would be able to identify HP2-1M in African samples. Thus, the assay design can be customized in accordance with the minor alleles present in a particular population. Our method may be useful for rapid and high-throughput genotyping of common *HP* alleles for association studies.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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Honoraria: None declared.
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Supplemental Table 1. Ct values of Hp5' and Hp2 and ΔCt obtained from 6.1 pg–100 ng genomic DNA with Hp^2/Hp^1, Hp^2/Hp^2

Hp genotype	*Genome DNA (ng)*	*Mean value ± SD of Ct of Hp5'*	*Range*	*Mean value ± SD of Ct of Hp2*	*Range*	*Mean value ± SD of ΔCt*	*Range*
Hp^2/Hp^1	0.0061	35.2 ± 0.33	34.7 - 35.5	37.1 ± 0.92	35.7 - 38.3	-1.85 ± 0.66	(-1.03) - (-2.93)
	0.024	33.0 ± 0.51	32.3 - 33.7	35.0 ± 0.56	34.1 - 35.8	-2.00 ± 0.58	(-1.11) - (-2.51)
	0.098	31.1 ± 0.20	30.9 - 31.5	33.0 ± 0.26	32.9 - 33.4	-1.91 ± 0.20	(-1.69) - (-2.24)
	0.39	29.3 ± 0.14	29.1 - 29.5	31.3 ± 0.17	31.1 - 31.6	-2.00 ± 0.07	(-1.87) - (-2.06)
	1.56	27.4 ± 0.12	27.3 - 27.6	29.5 ± 0.14	29.3 - 29.7	-2.06 ± 0.07	(-1.95) - (-2.12)
	6.25	25.5 ± 0.15	25.4 - 25.8	27.5 ± 0.14	27.4 -27.8	-2.02 ± 0.04	(-1.98) - (-2.08)
	25	23.6 ± 0.12	23.5 -23. 8	25.7 ± 0.13	25.6 -25.9	-2.07 ± 0.05	(-2.03) - (-2.16)
	100	21.7 ± 0.07	21.6 - 21.8	23.7 ± 0.08	23.6 - 23.9	-2.04 ± 0.05	(-1.99) - (-2.13)
Hp^2/Hp^2	0.0061	36.1 ± 0.61	35.7 - 36.9	36.9 ± 0.85	34.2 - 38.6	-0.87 ± 0.89	(0.47) - (-2.22)
	0.024	33.8 ± 0.40	33.3 - 34.4	34.6 ± 0.52	33.9 - 35.4	-0.81 ± 0.77	(0.57) - (-1.19)
	0.098	31.9 ± 0.22	31.6 - 32.1	32.8 ± 0.18	32.4 - 32.9	-0.89 ± 0.31	(-0.42) - (-1.22)
	0.39	30.0 ± 0.17	29.8 -30.2	30.8 ± 0.10	30.6 - 30.9	-0.79 ± 0.12	(-0.60) - (-0.91)
	1.56	28.0 ± 0.15	27.8 -28.2	28.8 ± 0.16	28.6 - 29.0	-0.79 ± 0.08	(-0.69) - (-0.90)
	6.25	26.0 ± 0.18	25.7 - 26.2	26.8 ± 0.17	26.5 - 27.0	-0.82 ± 0.03	(-0.78) - (-0.85)
	25	24.0 ± 0.16	23.8 - 24.2	24.9 ± 0.17	24.6 - 25.1	-0.91 ± 0.07	(-0.81) - (-1.01)
	100	22.2 ± 0.12	22.1 - 22.4	23.1 ± 0.16	22.8 -23.3	-0.85 ± 0.07	(-0.74) - (-0.90)

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Haptoglobin polymorphism in Mongolian population: Comparison of the two genotyping methods

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ABSTRACT

Background: Haptoglobin (Hp) polymorphisms have been suggested to be associated with many pathological conditions including cardiovascular diseases, infectious diseases, and type 2 diabetes.
Methods: We examined the association between *HP* genotypes and type 2 diabetes or anthropometric and clinical features in 946 Mongolians. *HP* genotypes were determined by two methods, TaqMan-based real-time PCR and conventional PCR. Hp phenotyping was also performed by polyacrylamide gel electrophoresis and peroxidase staining in a few selected samples.
Results: The 2 *HP* genotyping methods showed consistent results in 943 of 946 samples. Three samples that showed different results in the 2 PCR methods were determined to be heterozygous for the Hp Johnson allele by phenotyping. We did not find any association between *HP* genotypes and type 2 diabetes or the anthropometric and clinical features examined in the population studied.
Conclusions: TaqMan-based *HP* genotyping is reliable and will be useful for high-throughput association studies. Further studies of large numbers of subjects and functional differences in Hp types are needed.

1. Introduction

Haptoglobin (Hp) is a plasma glycoprotein highly expressed in the liver and is known to be one of the acute phase reactants [1]. It binds hemoglobin (Hb) to prevent both iron loss and kidney damage due to oxidative activity of Hb during intravascular hemolysis. Humans have a genetic polymorphism of the protein due to 2 codominant alleles, HP^1 and HP^2, that give rise to the three common phenotypes Hp1-1, Hp2-1, and Hp2-2 [2]. The Hp gene (*HP*) locates on the long arm of chromosome 16 (16q22.3) and consists of 5 (HP^1) or 7 (HP^2) exons [3,4]. HP^2 appears to have been generated by a 1.7-kb intragenic duplication of exons 3 and 4 of HP^1. Both HP^1 and HP^2 have been found in every population, although their frequencies vary considerably among populations [5].

Because of this polymorphic nature, Hp has been used as a genetic marker for identification of parentage and individuals, and several rare variants of the Hp phenotypes have been reported. These include Hp Johnson, Hp 2-1M, and anhaptoglobinemia [5]. The Hp Johnson phenotype results from the HP^3 allele, which has 3 tandem repeats of the same 1.7-kb DNA segment duplicated in HP^2 and comprises 9 exons [6]. The Hp 2-1M phenotype has been reported to have −61A>C base substitution in the promoter region, which seems to result in reduced expression of Hp2 relative to Hp1 [7,8]. The haptoglobin-gene deletion allele (HP^{del}) was identified by genetic analyses of an individual with anhaptoglobinemia and families having individuals with hypohaptoglobinemia [9]. HP^{del} has an approximately 28-kb deletion extending from the promoter region of *HP* to intron 4 of the haptoglobin-related gene [10]. The Hp Johnson phenotype has been observed worldwide with relatively low frequency, while Hp2-1M seems to be specific to African populations [5]. Although anhaptoglobinemia was observed among worldwide populations, the HP^{del} appears to be specific in East and Southeast Asian populations and a single causal mutation has been identified so far [10–14].

Hp polymorphisms have been reported to be associated with many pathological conditions such as infections, cardiovascular disease, obesity, and type 2 diabetes mellitus (DM); however, some studies have failed to detect these associations [5,15]. In the present study, we examined the association between *HP* genotypes and type 2 DM and the anthropometric and clinical features of a Mongolian population using conventional PCR and the recently developed TaqMan-based real-time PCR method.

2. Materials and methods

This study protocol was approved by the ethics committees of the Health Sciences University of Mongolia, the Ministry of Health of Mongolia, Mongolia, Jichi Medical University, and Kurume University, Japan.

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2.1. Subjects

All 946 subjects in this study were Mongolian people living in Ulaanbaatar City, and all provided informed consent to participate [16]. The 281 subjects with type 2 DM had already been diagnosed and were outpatients of the Diabetes Research Centre of Mongolia, Ulaanbaatar City, while 665 random blood donors without any history of DM were defined as the non DM subjects. Blood samples (5 ml) were drawn from the antecubital vein from all subjects. After measurement of blood glucose using Accu-Chek® (Roche Diagnostics, Basel, Switzerland), serum samples were separated and immediately frozen until analyzed at Jichi Medical University, Japan. The genomic DNA was extracted from the remaining white blood cells using a Puregene DNA purification kit (Gentra Systems, Inc., Minneapolis, MN). The DNA samples were stored and shipped at 4 °C. All kits and reagents were used according to their manufacturer's instructions.

2.2. HP genotyping

HP genotyping were determined using both conventional PCR and TaqMan-based real-time PCR methods. Amplification of the 1757-bp HP^1 allele-specific fragment was performed in a volume of 20 μl, containing 4 μl of 5× PrimeSTAR Buffer (Mg^{2+} plus) (Takara, Shiga, Japan), 250 nmol/l each of primers A and B [17], and about 0.1 to 10 ng genomic DNA. The temperature profile was 35 cycles of denaturing at 98 °C for 10 s and annealing and extension at 72 °C for 2 min. The HP^2 allele-specific fragment was amplified in a volume of 20 μl, 10 μl of Go taq® Green Master mix (Promega, Madison, WI), 250 nmol/l each of primers C and D [17], and about 0.1 to 10 ng genomic DNA. The temperature profile was 95 °C for 1 min, followed by 35 cycles of denaturing at 96 °C for 10 s, annealing and extension at 65 °C for 30 s, and then 1 final extension cycle at 72 °C for 1 min. Primers for the conventional PCR were synthesized by Hokkaido System Science (Sapporo, Hokkaido, Japan). HP^{del} zygosity was determined by duplex PCR using Hp-del-U, Hp-del-L, Hp-Ex1-U, and Hp-Ex1-L primers as described previously [10]. Resultant PCR products were size-fractionated by agarose gel electrophoresis.

In addition, TaqMan-based real-time PCR was performed as described previously [18,19]. Briefly the 20 μl PCR reaction contained about 0.1 to 10 ng genomic DNA, 10 μl of *Premix Ex Taq*™ (Perfect Real Time) (Takara), and the following primers and TaqMan probes at the indicated concentrations: HP2-F and -R primers (300 nmol/l), HP2 probe (83 nmol/l) for the detection of HP2, HP5′-F and -R primers (150 nmol/l), HP5′ probe (42 nmol/l) for the detection of the HP5′ region, HPdel-F and -R primers (300 nmol/l), and HPdel probe (83 nmol/l) for the detection of HP^{del}. The PCR temperature profile was 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing and extension at 60 °C for 30 s. All oligonucleotides for TaqMan-based real-time PCR were synthesized by Biosearch Technologies (Tokyo, Japan). Fluorescence was measured using an Mx3000P system (Agilent Technologies, inc., Tokyo, Japan) as described previously [18]. Data were collected and analyzed using MxPro™ Software (version 4.00, Agilent Technologies, Inc.). The ΔCt (difference in threshold cycles) of each sample was calculated as [Ct of HP2 (target)] − [Ct of HP5′ (control)]. The ΔΔCt of each sample (ΔΔCt sample) was obtained by [ΔCt of sample] − [ΔCt of reference] [20]. The ΔCt reference value was obtained using 5 ng of genomic DNA from an HP^2/HP^2 individual. The ratio of HP2:HP5′ of each sample was calculated as $2^{-\Delta\Delta Ct\ sample}$ [18].

2.3. Hp phenotyping

Plasma Hp phenotypes were determined by continuous polyacrylamide gel (5–20%, 120 × 100 mm, e-PAGEL®, Atto, Tokyo, Japan) electrophoresis (PAGE) of Hb-supplemented plasma using 0.01 mol/l Tris and 0.077 mol/l glycine (200 V for 2.5 h). After electrophoresis, the gel was treated with 0.2 g leucomalachite green and 1 g zinc powder in 50 ml of 40% acetic acid solution. The stain was activated with 3% hydrogen peroxide [13].

2.4. Statistical analysis

Data are expressed as the means ± SD, median (range) or proportions for categorical variables. Hardy–Weinberg equilibrium was calculated using the chi-square test. Categorical valuables were compared using Fisher's exact test. One-way analysis of variance (ANOVA) was used to compare any differences between continuous valuables. Nonparametric data were analyzed by the Kruskal–Wallis test. Associations between the *HP* genotypes and type 2 DM, body mass index (BMI), blood pressure, and serum lipid values were evaluated. A $P<0.05$ was considered to be statistically significant.

3. Results

3.1. HP genotyping

We determined the *HP* genotypes using TaqMan-based real-time PCR, and the results were compared with those obtained from a conventional PCR method. The recently developed real-time PCR method is based on comparative threshold cycles (Ct) of the HP^2-specific sequence (duplication junction) and a control sequence (5′ flanking sequence of exon 1 of *HP*). This method was validated by analyzing about 130 samples [18]. Real-time PCR was performed by duplicate measurements for each sample. In 943 of 946 samples, the results obtained from the TaqMan-based method were consistent with those from conventional PCR. In these 943 samples, the Ct values of the control sequence (Hp5′) ranged from 20.3 to 29.4. The ΔCt value of HP^2/HP^2 reference was 0.6, and the $2^{-\Delta\Delta Ct\ sample}$ values of each genotype were shown in Table 1. Seventy samples with no Hp2 signal were typed as HP^1/HP^1. Samples with amplification of the Hp5′ and HP^{del} allele-specific regions and without amplification of the HP^2 allele-specific region were genotyped as HP^1/HP^{del} (5 individuals), while those with all three signals were genotyped as HP^2/HP^{del} (12 individuals). We did not detect a HP^{del} homozygote, a sample with only amplification of HP^{del}, in this study.

In three samples, however, the results from the two methods were discordant. In two of them, the genotype determined by the TaqMan-based method was HP^2/HP^2 (mean $2^{-\Delta\Delta Ct\ sample}$ value: 1.02, $n=2$), while that by conventional PCR was HP^1/HP^2. In one of these, the copy number relative to the 5′ sequence of the Hp2-specific sequence ($2^{-\Delta\Delta Ct\ sample}$ value) was about 1.36, while those of 36 HP^1/HP^2 ranged between 0.44 and 0.54 [mean (SD): 0.49 (0.03)] and those of 46 HP^2/HP^2 ranged between 0.90 and 1.07 [mean (SD): 1.00 (0.04)] in the same 96-well assay plate. However, the genotype of this sample determined by conventional PCR was HP^2/HP^2. We previously suggested that the main limitation of the present TaqMan-based real-time PCR method, in comparison to Southern blotting, phenotyping, and conventional PCR methods, is misidentification

Table 1
HP genotyping of 946 Mongolian samples based on TaqMan assay.

HP genotype	Number of individuals	Total mean value ± SD of $2^{-\Delta\Delta Ct\ sample}$ (Hp2:Hp5′)	Range
HP^1/HP^1	70	0 ± 0	0–0
HP^1/HP^2	363	0.49 ± 0.04	0.38–0.64
HP^2/HP^2	493	1.00 ± 0.08	0.76–1.27
HP^1/HP^{del}	5	0 ± 0	0–0
HP^2/HP^{del}	12	1.01 ± 0.10	0.92–1.29
HP^1/HP^3	2	1.02	0.98–1.06
HP^2/HP^3	1	1.36	1.36

Table 2
Clinical characteristics and *HP* genotypes of type 2 DM subjects.

	HP genotype				
	HP^{1}/HP^{1}	HP^{1}/HP^{2}	HP^{2}/HP^{2}	Others	*P* value
Male/female (*N*)	13/14 (27)	53/45 (98)	75/74 (149)	3/4 (7)	NS[a]
Age (y)	50.9 ± 11.0	51.3 ± 9.8	53.2 ± 9.1	54.4 ± 14.5	NS
Body mass index (kg/m^2)	29.6 ± 5.2	29.7 ± 5.7	29.6 ± 5.5	27.3 ± 2.9	NS
Body fat percentage (%)	37.4 ± 10.2	36.7 ± 9.2	37.1 ± 10.9	35.3 ± 9.8	NS
Waist-to-hip ratio	0.92 ± 0.08	0.94 ± 0.08	0.93 ± 0.08	0.91 ± 0.10	NS
Systolic blood pressure (mmHg)	144.8 ± 26.3	145.7 ± 27.6	147.6 ± 29.1	128.6 ± 14.4	NS
Diastolic blood pressure (mmHg)	87.8 ± 15.5	87.0 ± 15.5	88.1 ± 14.3	80.4 ± 11.5	NS
Total cholesterol (mg/ml)	200.9 ± 46.7	192.8 ± 39.9	190.6 ± 40.8	184.8 ± 30.7	NS[c]
HDL cholesterol (mg/ml)	50.1 ± 12.6	49.3 ± 11.6	49.5 ± 10.4	49.4 ± 13.3	NS[c]
Triglyceride (mg/ml)	132 (39–1740)	164 (37–1480)	135 (49–1161)	112 (70–168)	NS[b,c]
Fasting blood glucose (mmol/l)	12.1 (5.3–18.9)	11.1 (4.4–27.5)	10.4 (2.7–24.8)	8.6 (7.3–13.6)	NS[b,c]
Fasting insulin (mU/ml)	16.8 (5.2–88.8)	18.9 (1.6–85.5)	18.1 (1.6–121.0)	16.1 (1.5–29.0)	NS[b,d]
Adiponectin (ng/ml)	21.3 (4.4–86.6)	18.9 (3.1–267.6)	17.6 (1.0–220.6)	13.3 (5.2–38.5)	NS[b,c]
Fructosamine (mmol/ml)	323 (153–516)	368 (225–589)	343 (216–709)	366 (248–529)	NS[b,c]

Data are means ± SD, medians (ranges) for skewed variables, or proportions for categorical variables. *P* values derived from one-way ANOVA unless annotated.
Others (2 HP^{1}/HP^{del}, 4 HP^{2}/HP^{del} and 1 HP^{1}/HP^{3}) were excluded from the analyses.
[a] Fisher's exact test.
[b] Kruskal–Wallis test.
[c] $N=17$ (HP^{1}/HP^{1}), 57 (HP^{1}/HP^{2}), 104 (HP^{2}/HP^{2}), 5 (Others). Others (1 HP^{1}/HP^{del}, 3 HP^{2}/HP^{del} and 1 HP^{1}/HP^{2}) were excluded from the analyses.
[d] $N=16$ (HP^{1}/HP^{1}), 57 (HP^{1}/HP^{2}), 104 (HP^{2}/HP^{2}) , 5 (Others). Others (1 HP^{1}/HP^{del}, 3 HP^{2}/HP^{del} and 1 HP^{1}/HP^{2}) were excluded from the analyses.

of rare variants such as Hp Johnson because of the principle of the method [18]. To examine this possibility, we performed Hp phenotyping on the three samples using polyacrylamide gel electrophoresis. These three individuals had heterozygous Hp Johnson (HP^{n}) alleles: two individuals were Hp1-3 and one individual was Hp2-3 (data not shown). Thus, the *HP* genotypes of 946 Mongolians were 70 HP^{1}/HP^{1}, 363 HP^{1}/HP^{2}, 493 HP^{2}/HP^{2}, 5 HP^{1}/HP^{del}, 12 HP^{2}/HP^{del}, 2 HP^{1}/HP^{3}, and 1 HP^{2}/HP^{3}, and the allele frequencies were $HP^{1}=0.27$, $HP^{2}=0.72$, $HP^{del}=0.009$, and $HP^{3}=0.0016$. The allele and genotype frequencies in this study were quite similar to those of Mongolians of a previous study [21]. The genotype frequencies were in Hardy–Weinberg equilibrium in both type 2 DM ($P=0.433$) and non DM ($P=0.970$) subjects.

3.2. Association of *HP* genotypes and type 2 DM, anthropometric and clinical features

Tables 2 and 3 show the sex, age, body mass index, body fat percentage, waist/hip ratio, and systolic and diastolic blood pressure of each *HP* genotype of type 2 DM ($N=274$, we excluded 7 sample having the HP^{3} and HP^{del} alleles from the analyses, Table 2) and of healthy subjects ($N=652$, we excluded 13 samples having the HP^{3} and HP^{del} alleles from the analyses, Table 3). We did not find any association between these parameters and *HP* genotypes in both groups. We also indicated the relationship of several biochemical variables including fasting blood glucose, total cholesterol, triglyceride, HDL cholesterol, fructosamine, fasting insulin, and adiponectin to each *HP* genotype ($N=182$–452). However, we did not detect any association between these parameters and *HP* genotypes either. In addition, the associations between *HP* genotype and allele frequencies and type 2 DM were examined using Fisher's exact test. However, we did not find any association ($P=0.313$, between genotypes and DM; $P=0.766$, allele frequencies and DM).

4. Discussion

Because the HP^{2} allele has 3 tandem repeats of the same 1.7-kb DNA segment duplicated in the HP^{1} allele [6], 2 copies of Hp2 primers and probe-binding sites of the present TaqMan-based real-time PCR are presented in one HP^{3}. As predicted previously, we judged two HP^{1}/HP^{3} individuals as HP^{2}/HP^{2}, while we obtained the relative copy number of

Table 3
Clinical characteristics and *HP* genotypes of non DM subjects.

	HP genotype				
	HP^{1}/HP^{1}	HP^{1}/HP^{2}	HP^{2}/HP^{2}	Others	*P* value
Male/female (*N*)	15/28 (43)	119/146 (265)	134/210 (344)	8/5 (13)	NS[a]
Age (y)	47.6 ± 11.4	50.1 ± 10.6	49.4 ± 10.4	52.2 ± 8.4	NS
Body mass index (kg/m^2)	26.3 ± 4.6	26.9 ± 4.9	26.6 ± 4.5	29.0 ± 6.1	NS
Body fat percentage (%)	36.2 ± 12.6	33.7 ± 9.8	34.7 ± 9.6	36.9 ± 8.6	NS
Waist-to-hip ratio	0.80 ± 0.10	0.82 ± 0.11	0.82 ± 0.11	0.80 ± 0.17	NS
Systolic blood pressure (mmHg)	132.6 ± 22.6	131.5 ± 22.6	130.0 ± 23.1	136.4 ± 34.3	NS
Diastolic blood pressure (mmHg)	83.8 ± 13.3	84.0 ± 14.4	83.6 ± 15.5	85.0 ± 17.7	NS
Total cholesterol (mg/ml)	176.8 ± 37.4	176.8 ± 34.1	180.4 ± 38.3	203.8 ± 42.1	NS[c]
HDL cholesterol (mg/ml)	53.3 ± 9.6	54.9 ± 11.5	53.8 ± 11.7	56.5 ± 9.4	NS[c]
Triglyceride (mg/ml)	101 (32–568)	87 (30–1210)	96 (34–635)	134 (67–292)	NS[b,c]
Fasting blood glucose (mmol/l)	5.6 (4.6–6.8)	5.5 (4.2–6.9)	5.5 (4.2–6.8)	5.6 (4.4–6.6)	NS[b,c]
Fasting insulin (mU/ml)	11.6 (2.5–213.0)	11.7 (1.5–87.7)	11.3 (1.0–161.0)	12 (2.86–44.0)	NS[b,c]
Adiponectin (ng/ml)	33.8 (4.3–85.4)	32.3 (2.0–249.5)	34.0 (3.0–103.2)	27.9 (4.81–66.9)	NS[b,d]
Fructosamine (mmol/ml)	238 (206–288)	236 (191–340)	236 (194–307)	227 (205–255)	NS[b,c]

Data are means ± SD, medians (ranges) for skewed variables, or proportions for categorical variables. *P* values derived from one-way ANOVA unless annotated.
Others (3 HP^{1}/HP^{del}, 8 HP^{2}/HP^{del}, 1 HP^{1}/HP^{3} and 1 HP^{2}/HP^{3}) were excluded from the analyses.
[a] Fisher's exact test.
[b] Kruskal–Wallis test.
[c] $N=28$ (HP^{1}/HP^{1}), 181 (HP^{1}/HP^{2}), 237 (HP^{2}/HP^{2}), 11 (Others). Others (3 HP^{1}/HP^{del}, 6 HP^{2}/HP^{del}, 1 HP^{1}/HP^{3} and 1 HP^{2}/HP^{2}) were excluded from the analyses.
[d] $N=28$ (HP^{1}/HP^{1}), 179(HP^{1}/HP^{2}), 235 (HP^{2}/HP^{2}), 11 (Others). Others (3 HP^{1}/HP^{del}, 6 HP^{2}/HP^{del}, 1 HP^{1}/HP^{3} and 1 HP^{2}/HP^{2}) were excluded from the analyses.

1.36 in one HP^2/HP^2. However, we examined only one HP^2/HP^2 individual, and because the phenotyping was performed on only a few selected samples, we will phenotype further cases of HP^2/HP^2 to evaluate whether the TaqMan real-time PCR method can detect this genotype accurately. We judged 2 HP^1/HP^2 individuals as HP^1/HP^2 and 1 HP^2/HP^2 as HP^2/HP^2 by the conventional PCR method in the present amplification condition. This misclassification is due to a failure of amplification of a 5.1-kb fragment corresponding to HP^2 by conventional PCR using primers A and B. In fact, we could not amplify a 3.4-kb fragment corresponding to HP^2 in many HP^1/HP^2 samples using primers A and B (data not shown). Thus, the present PCR method seemed to have difficulty amplifying both HP^2 and HP^2 fragments using primers A and B, although we could amplify the 349-bp HP^2-specific sequence of both HP^2 and HP^2 alleles using primers C and D. The present results further supported the supposition that the TaqMan-based real-time PCR method is reliable because the results showed perfect concordance with those of conventional PCR in 943 genotypes, except for the 3 individuals with HP^2. This method may be useful for rapid and high-throughput genotyping of common *Hp* alleles for association studies. In addition, we detected HP^{del}, which is a causal mutation of severe anaphylactic transfusion reactions due to production of anti-Hp antibodies, using this method. In the present study, we found three HP^2 alleles among 946 individuals; thus, the allele frequency of HP^2 is estimated to be 0.0016. Previous studies estimated the allele frequency of HP^2 to be 0.00015 in a Japanese population and 0.0018 in a Melanesian population [22,23]. Thus, the allele frequency of HP^2 in a Mongolian population seems to be similar to that in Melanesians and is relatively high. In addition, we found 17 HP^{del} alleles in the 946 individuals, and the allele frequency was calculated to be 0.009. This value is similar to previous results obtained in a Mongolian population (0.008; 2 heterozygotes in 119 individuals) [12].

In the present study, we did not find any association between *HP* genotypes and type 2 DM or the anthropometric and clinical features examined in a Mongolian population. However, we did not evaluate the association of rare variants of *HP* including HP^{del} and HP^2 because of the relatively low frequencies of these alleles. The HP^2 allele was suggested to be associated with an increased risk for the development of diabetic nephropathy in Israeli and Egyptian populations [24,25]. In addition, the Hp 2-2 phenotype was reported to be a risk factor for type 2 DM in a Ghanaian population and gestational DM in an Australian population [26,27]. The Hp phenotype and genotype have also been associated with susceptibility to defective lipid metabolism, malignancy, cardiovascular diseases, inflammatory diseases, infection, nonalcoholic fatty liver disease, and autoimmune diseases [5,28–32]. Such associations may be explained by functional differences between the subtypes of Hb binding and its rate of clearance from the plasma. Recent studies identified profound differences between the antioxidant and immunomodulatory properties of Hp1 and Hp2 polypeptides [15]. However, there are also corresponding reports of conflicting or negative disease associations [33–36]. These controversial results might be due to the ethnic backgrounds of studied populations. Alternatively such associations might be marginal [5]. To resolve these problems, further analyses using massive and worldwide population samples are required. Thus, the TaqMan-based real-time PCR method may be useful for rapid and high-throughput genotyping of common *Hp* alleles for the purposes of association study.

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Development and validation of a SYBR Green I–based real-time polymerase chain reaction method for detection of haptoglobin gene deletion in clinical materials

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BACKGROUND: Anhaptoglobinemic patients run the risk of severe anaphylactic transfusion reaction because they produce serum haptoglobin (Hp) antibodies. Being homozygous for the Hp gene deletion (HP^{del}) is the only known cause of congenital anhaptoglobinemia, and clinical diagnosis of HP^{del} before transfusion is important to prevent anaphylactic shock. We recently developed a 5′-nuclease (TaqMan) real-time polymerase chain reaction (PCR) method.

STUDY DESIGN AND METHODS: A SYBR Green I–based duplex real-time PCR assay using two forward primers and a common reverse primer followed by melting curve analysis was developed to determine HP^{del} zygosity in a single tube. In addition, to obviate initial DNA extraction, we examined serially diluted blood samples as PCR templates.

RESULTS: Allelic discrimination of HP^{del} yielded optimal results at blood sample dilutions of 1:64 to 1:1024. The results from 2231 blood samples were fully concordant with those obtained by the TaqMan-based real-time PCR method.

CONCLUSION: The detection rate of the HP^{del} allele by the SYBR Green I–based method is comparable with that using the TaqMan-based method. This method is readily applicable due to its low initial cost and analyzability using economical real-time PCR machines and is suitable for high-throughput analysis as an alternative method for allelic discrimination of HP^{del}.

Anaphylaxis is a severe nonhemolytic transfusion reaction, and determination of its causes is urgently needed.[1,2] The absence of a serum protein such as immunoglobulin A or haptoglobin (Hp) is one factor that can lead to anaphylactic transfusion reactions[1-4] due to production of serum antibodies against it.[5-7] At present, a homozygous deletion of the Hp gene (HP^{del}) is the only known cause of anhaptoglobinemia.[5,8]

Hp binds hemoglobin (Hb) to prevent both iron loss and kidney damage during hemolysis. Humans have a genetic polymorphism of the protein due to two codominant alleles, Hp1 and Hp2, that give rise to the three major phenotypes, Hp1, Hp2-1, and Hp2.[9,10] Because of its polymorphic nature, Hp has been used as a genetic marker for identification of individuals and determination of parentage. Anomalous inheritance of the Hp phenotypes was encountered during determinations of parentage, and HP^{del} was identified by genetic analyses of one such family in Japan.[8]

The HP^{del} allele lacks an approximately 28-kb segment of Chromosome 16 extending from the promoter region of the Hp gene to Intron 4 of the Hp-related gene (*HPR*).[5] Two different methods have been demonstrated to be useful for the detection of anhaptoglobinemia. One is detection

ABBREVIATIONS: Hp = haptoglobin; T_m = melting temperature.

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of HP^{del} allele by polymerase chain reaction (PCR) or Southern blotting,[8] and the other is quantification of Hp protein by enzyme-linked immunosorbent assay (ELISA).[11] The ELISA method is sensitive and able to discriminate efficiently between anhaptoglobinemia and hypohatoglobinemia. However, this method requires confirmation of the presence of HP^{del} by a PCR-based method. A duplex PCR method allows determination of the zygosity of HP^{del}. Using this method, frequencies of the HP^{del} allele were examined in several human populations. This allele has been found only in East and Southeast Asian populations (Chinese, Korean, Japanese, Mongols, Thais, and Indonesians) but not in African, West and South Asian, and European populations so far.[5,12-15] Detection of homozygotes for HP^{del} before blood transfusion or blood component infusion is important to prevent severe side effects of transfusion, because washed red blood cells and platelet concentrate are effective in preventing the transfusion-related anaphylactic reactions.[16]

Although isolation of genomic DNA, conventional PCR, and gel electrophoresis are routine methods in research and molecular biology laboratories, they are not suitable for large-scale analysis or diagnosis before transfusion in the clinical laboratory because they require laborious post-PCR processing steps. Real-time PCR is a high-throughput, rapid, and sensitive method that has become common.[17] It also eliminates post-PCR processing of PCR products, which reduces the chance of carry-over contamination. Recently we established a diagnostic method for detection of HP^{del} by a 5′-nuclease assay using dual-labeled (TaqMan) probes.[18] This method is highly specific due to the sequence-specific hybridization of the probe and is cost-effective when many samples are treated simultaneously, such as screening for anhaptoglobinemic patients in a blood donor pool or for large-scale screening in various populations. However, if only a few samples are examined in the clinical laboratory, the initial cost is high because two dual-labeled probes are necessary for determination of zygosity. In addition, multichannel real-time PCR machines, which are expensive compared to single-channel real-time PCR machines, are required to perform this system.

To resolve this problem, we developed a SYBR Green I–based real-time PCR method for detection of HP^{del}. SYBR Green I, an intercalating dye that binds to double-stranded DNA, is used to detect the accumulated PCR product.[17] Because an increase in the fluorescent signal is detected not only by specific amplification of the product but also by the primer-dimer or nonspecific amplified product, dissociation curve analysis is required to confirm the specificity of the PCR product. In addition, to reduce the time and cost of genomic DNA isolation, we examined serially diluted blood samples as PCR templates instead of genomic DNA.

MATERIALS AND METHODS

This study protocol was approved by the ethics committee of Kurume University School of Medicine. Fresh venous blood samples from patients who were scheduled for blood transfusion at Kurume University Hospital were used. The whole blood samples were collected in ethylenediaminetetraacetate (EDTA)- or heparin-containing tubes. We also used genomic DNA samples with known HP^{del} genotypes.

PCR was performed using the following three primers (two forward primers and one common reverse primer): Hpdel-F (5′-TATTTCTTTATGGCACTGGGGAACA-3′, sequence from 690 to 714 bp in GeneBank No. AB025320) for amplification of the regions encompassing the HP^{del} breakpoint, Hpr-F (5′-CTGCAACTATTGGAAATGAG ATCAGC-3′, sequence from 12,920 to 12,945 bp in GeneBank No. NC_000016.9, located in the 3′ end of the deleted region in HP^{del}, Intron 4 of the *HPR*) for amplification of *HPR* Intron 4, and the common reverse primer Hpdel-R (5′-GAGCAAGACACTCGTGAGTGGAAAT-3′, reverse sequence from 823-799 bp of GeneBank No. AB025320 and reverse sequence from 13,067 to 13,043 bp of GeneBank No. NC_000016.9). The HP^{del} breakpoint occurs between 782 and 783 bp in the sequence of GeneBank AB025320. The positions of the three primers are presented in Fig. 1. All primers were designed using computer software (Primer 3, http://primer3.sourceforge.net/).[19]

The amplification was performed in a volume of 20 μL containing 10 μL of 2× SYBR Premix Ex Taq II (perfect real time; Takara, Shiga, Japan), 75 nmol/L Hpr-F primer, 500 nmol/L Hpdel-690F, 250 nmol/L Hpdel-823R primer, and 1 μL of the template (severalfold diluted fresh venous whole blood or genomic DNA). Wells without a template were included as negative controls. For determination of optimal PCR conditions, amplifications were performed using a 96-well thermal cycler (Veriti, Life Technologies Japan, Tokyo, Japan). Determined optimal conditions in the real-time PCR are as follows: 1) place the 96-well plate in a real-time PCR system (Mx3000P, Agilent Technologies, Tokyo, Japan); 2) heat the plate at 95°C for 30 seconds; 3) repeat the following for 40 cycles, 95°C for 5

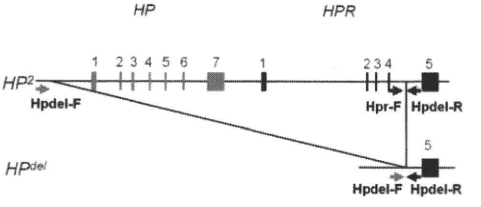

Fig. 1. Structures of the HP^2, and HP^{del} alleles and relative positions of the Hpdel-F, Hpr-F, and Hpdel-R primers.

seconds, and 65°C for 30 seconds; and 4) melting curve analysis performed as follows: heat the plate at 95°C for 1 minute, rapid cooling to 70°C, 70°C for 30 seconds, and heat the plate at 0.2°C/second to 90°C. The accumulation of PCR products and melting curve analysis were monitored by measurement of the level of SYBR Green I fluorescence with excitation/emission filters of 492/516 nm (using an FAM filter set).

After the dissociation curve analysis, we determined the length of PCR products using a microchip electrophoresis system (MCE-202 MultiNA, Shimadzu, Kyoto, Japan). In addition to SYBR Green I real-time PCR method, the zygosity of the HP^{del} allele was also determined in parallel using TaqMan-based real-time PCR method as described previously.[18]

RESULTS AND DISCUSSION

Duplex real-time PCR of HP^{del} and HPR

We investigated duplex real-time PCR, which permits discrimination of HP^{del} zygosity in a single tube, to determine if the melting temperature (T_m) values of the two PCR products are sufficiently different to distinguish them from each other. In the previously reported conventional PCR method, the lengths of the PCR products were 476 and 315 bp for detection of *HP* Exon 1 and HP^{del}, respectively.[5] These lengths are suitable and convenient for gel electrophoresis but too long to amplify in the buffer containing SYBR Green I, particularly when using a blood sample directly as a template. Thus, we planned to amplify smaller PCR products in the present SYBR Green I–based real-time PCR method. For detection of the nondeleted allele, we selected the 3′ end of the deleted region (Hpr-F; 107-82 bp upstream of the 3′ breakpoint of the HP^{del}) as a forward primer and reduced the length of the PCR product to 148 bp using a Hpdel-R primer as a reverse primer. We also reduced the length of the PCR product of the HP^{del} breakpoint to 134 bp using Hpdel-F as a forward primer and Hpdel-R.

To evaluate this real-time PCR method, genomic DNAs whose genotypes were already known to be lacking HP^{del} (*HP/HP*), heterozygous for HP^{del} (*HP/HP*^{del}), and homozygous for HP^{del} (HP^{del}/HP^{del}) were used as templates. We failed to establish an applicable duplex PCR method with similar peak intensities for HP^{del} and *HPR* signals when the three primers were adjusted to the same concentration (100-500 nmol/L). This is probably due to the apparent preferential binding of SYBR Green I to selected amplicons, as suggested previously.[20] Accordingly, we used several different ratios of concentrations of the three primers and obtained almost similar peak intensities for HP^{del} and *HPR* signals when we used 75 nmol/L Hpr-F, 500 nmol/L Hpdel-F, and 250 nmol/L Hpdel-823R primers. The annealing temperatures and number of cycles for two PCR assays for allelic discrimination of HP^{del} using blood samples were optimized to determine the PCR condition that gave the best specificity without a reduction in yield. The optimal annealing and extension temperature and numbers of cycles for both amplifications were found to be 65°C and 40 cycles, respectively (data not shown).

An increase in the fluorescent signal in the amplification plot was observed in all three samples tested. The dissociation curve analysis revealed a single peak (T_m = 84.3-84.7°C) corresponding to 148 bp of *HPR* Intron 4 product in genomic DNA samples from *HP/HP* individuals. Two different peaks (T_m = ~80.3 and 84.5°C) corresponding to the 134-bp HP^{del} product and *HPR* were observed in the genomic DNA sample from *HP/HP*^{del} individuals, while a single peak (T_m = 80.3-80.7°C) corresponding to the HP^{del} was observed in genomic DNA sample from the HP^{del}/HP^{del} individual (Fig. 2). After the dissociation curve analysis, microchip electrophoresis using MCE-202 MultiNA was performed and the characteristic length of PCR products for *HPR* Intron 4 at 148-bp (estimated sizes by microchip electrophoresis were between 156-159 bp) and the HP^{del} product at 134 bp (estimated sizes by microchip electrophoresis were between 140-142 bp) were detected (data not shown). No increase in the fluorescent signal was detected in the negative control (data not shown).

Evaluation of templates and plates

We then applied this method using a blood sample as a template. We first examined the effects of the anticoagulants in blood collecting tubes and dilutions of blood samples on the efficiency of PCR amplification. Blood was diluted with PCR grade water (later, we used 50 mmol/L NaOH instead of water, as mentioned below). Like the TaqMan real-time PCR method,[18] the SYBR Green I real-time PCR method worked well on blood samples collected in EDTA-containing tubes using 1 µL of undiluted and 1:4, 1:16, 1:64, 1:256, 1:1024, and 1:4096 dilutions of the whole blood sample as a template. The Ct values were not proportional to the amount of the blood template (Fig. 3). This is partly because both the fluorescence and the amplification seemed to be inhibited by blood component(s) such as Hb. Specific PCR products were observed in all blood samples diluted up to 1:1024 but not in the 1:4096 dilution. Instead, undesired PCR products with the T_m value of 76.8°C (as described below) were occasionally observed in 1:4096 dilution samples.

We also used heparin-containing tubes and observed almost identical results for the same serial dilutions of blood samples as those of EDTA-containing tubes (data not shown). These results suggested that blood sample dilutions between 1:64 and 1:1024 collected in both EDTA- and heparin-containing tubes seemed to provide a good template for this real-time PCR method. Thus, we used

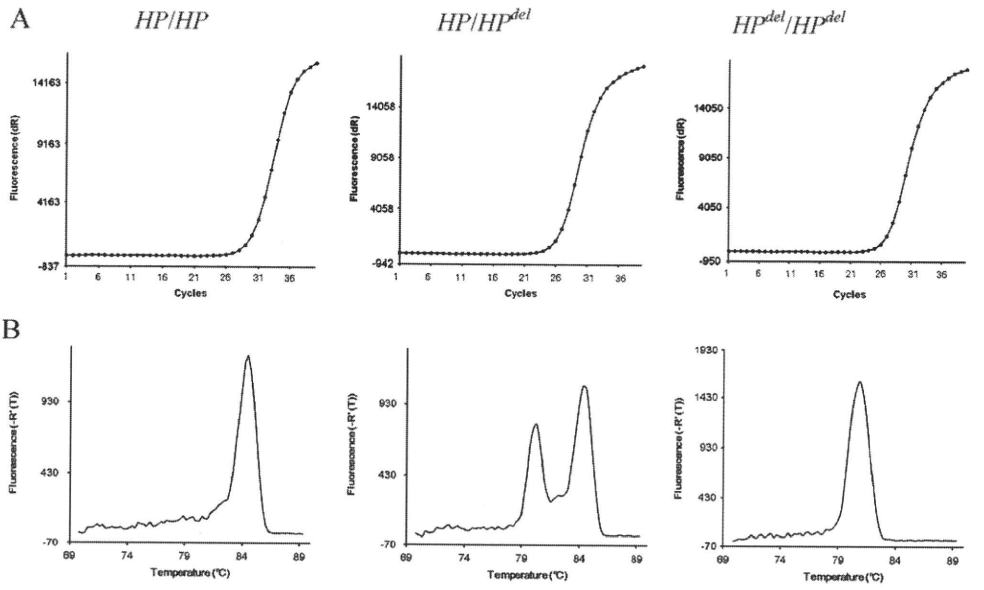

Fig. 2. SYBR Green I–based real-time PCR analysis for detection of *HP*^{del} and *HP*. Genomic DNA whose genotypes are *HP/HP* (left), *HP/HP*^{del} (middle), and *HP*^{del}/*HP*^{del} (right) were used as templates. (A) Duplex real-time amplification plot for *HPR* (intron 4) and *HP*^{del} as targets. (B) Dissociation curves provide a graphic representation of the PCR product after the amplification process. A single peak or two peaks with different T_m values were observed. T_m values of *HPR* products were approximately 84.5°C, and those of *HP*^{del} were approximately 80.3°C.

1:100 dilutions of blood samples as templates for further studies. We also compared the results of PCR amplification using frozen blood (obtained in EDTA-containing tubes) diluted 1:100 to freshly drawn blood samples as templates. We observed almost identical PCR amplification in both samples (data not shown). In addition, this method can be applied to blood samples that were collected from patients and stored for at least 10 days at 4°C.

We examined the effects of type of PCR plates on the fluorescent signal. A white PCR plate (Sorenson ultraAmp PCR semiskirted 96-well white plates, Nippon Genetics, Tokyo, Japan) increased the fluorescent signal approximately threefold compared with a transparent one (data not shown).

Validation of the method

We then examined blood samples from 2231 patients who were scheduled for blood transfusion at Kurume University Hospital. Diagnosis of the *HP*^{del} allele was performed in parallel using the TaqMan-based real-time PCR method.[18] The real-time PCR method was performed in duplicate assays for each of 45 samples using a white 96-well plate in both the SYBR Green I and the TaqMan-based methods. During this large-scale experiment, fluorescent signals of some samples (particularly in TaqMan-based real-time PCR method) were too small to be detected for presently unknown reason(s). This phenomenon was not reproducible; that is, although the signal of one sample was too small to detect, another well of a duplicate sample showed adequate signal strength. To resolve this problem, blood was diluted to 100-fold with 50 mmol/L NaOH instead of water, treated at 95°C for 5 minutes, and then used as a template. This procedure seemed to improve the signal strength significantly, although signals of some samples seemed to be still a little reduced. The results of duplex assays of the present SYBR Green I real-time PCR method were completely identical to those of the TaqMan-based real-time PCR method[18] (i.e., 2163 individuals with *HP/HP*, 67 individuals with *HP/HP*^{del}, and one *HP*^{del}/*HP*^{del}). Accordingly, the frequency of *HP*^{del} is calculated to be 0.015 in the Kyushu area (western Japan), and the incidence of individuals homozygous for the *HP*^{del} was expected to be 1 in every 4200 individuals. When we used blood samples treated with