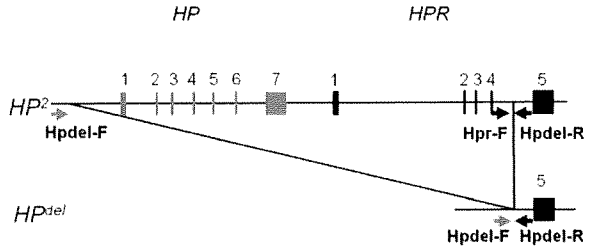
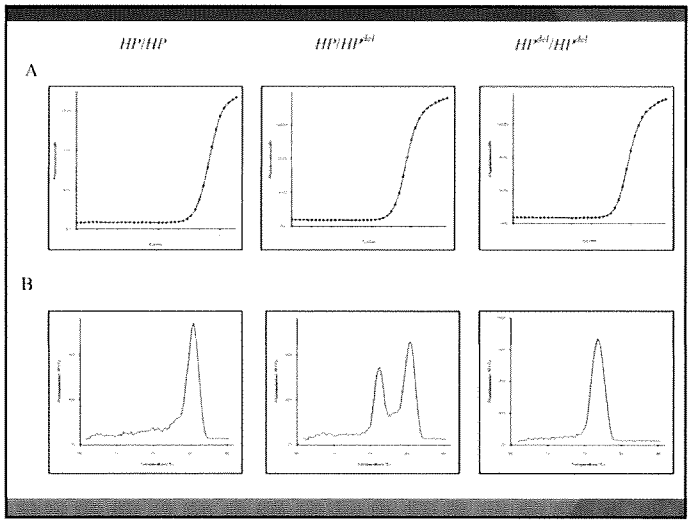
*HP, HPR*の構造 (*Hp*^{2}および*Hp*^{del} alleles)

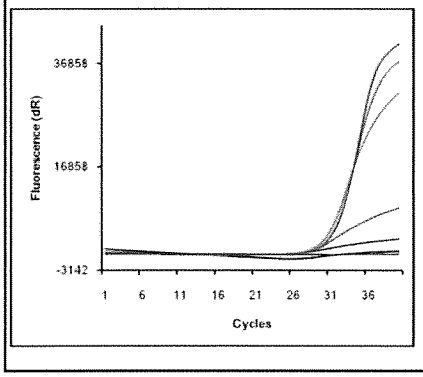


方法

- 10 μl of 1 x SYBR® *Premix Ex Taq*™ II (Perfect Real Time) (Takara), 75 nM Hpr-F primer, 500 nM Hpdel-690F, 250 nM Hpdel-823R primer, 0.5 μl of the template (血液ないしはゲノムDNA)
- 95°C, 30 s ➡ 95°C, 5 s and 65°C,30 s (40 cycles) ➡ 95°C,1 min, 70°C, 30 s ➡ heat the plate at 0.2°C/s to 90°C
- Tm解析: Mx3000P real-time PCR system, FAM filter set (Agilent)

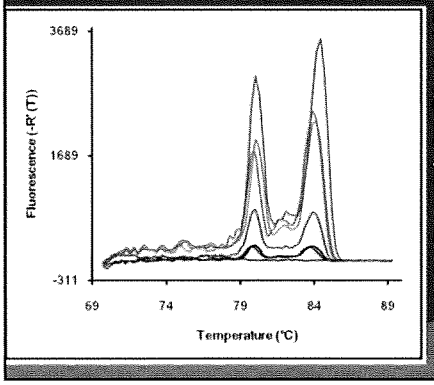


血液を直接鋳型にした場合の増幅曲線



濃紺(希釈なし)
赤 (4倍希釈)
緑 (16倍希釈)
灰 (64倍希釈)
黄緑 (256倍希釈)
青 (1024倍希釈)
紫 (4096倍希釈)

血液を直接鋳型にした場合の融解曲線



濃紺(希釈なし)
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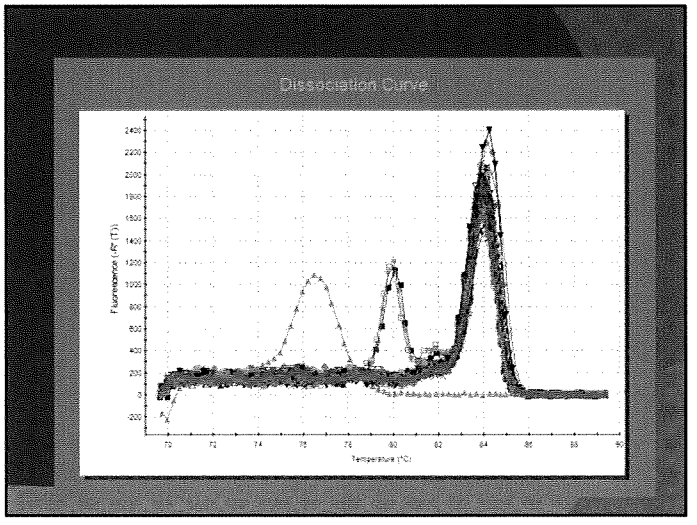
緑 (16倍希釈)
灰 (64倍希釈)
黄緑 (256倍希釈)
青 (1024倍希釈)
紫 (4096倍希釈)



輸血予定患者の遺伝子検査

久留米大学病院臨床検査部（部長佐川公矯教授）において本法の有用性を検定するために輸血予定患者の遺伝子検査を実施中。

2437人の検査が終了（平成21年1月から平成21年12月末）

75の*Hp*^{del} allelesを検出（73人のヘテロ接合体と1人のホモ接合体）。

遺伝子頻度は75/4874 = 0.00154

Hp^{del}のホモ接合体の頻度は約4200人に1人と推定。

SYBR Greenを用いたreal time PCRによる*Hp*^{del}診断法

- 2437検体の全例でTaqMan法の結果と一致。
- Duplicateで行った実験結果もすべて一致。
- primerのみでprobeを必要としないためコストの削減が可能。
- TaqMan法に比べて血液を直接鋳型にした場合のシグナル抑制が認められる頻度が低い。
- 臨床診断に十分利用可能。
- Negative controlにPrimer dimer が認められる場合がある。（現在試薬キットを変更して検討中）

厚生労働科学研究費補助金

（医薬品・医療機器等レギュラトリーサイエンス総合研究事業）

分担研究報告書

「輸血副作用の原因遺伝子ハプトグロビン欠失アリルの

迅速簡便な診断法の確立と輸血前診断への臨床応用」に関する研究

研究分担者　佐川　公矯　久留米大学　医学部　教授

研究要旨: 輸血後アナフィラキシーショックの原因の一つである血漿蛋白欠損のうち、日本人を含めた東アジア人集団ではハプトグロビン欠損症が最も高頻度であることが知られている。そのため2005年度から各種血液製剤の添付資料の「慎重投与の項」に記載されるようになった。本研究計画は、ハプトグロビン欠損症のうち先天性無ハプトグロビン血症の原因遺伝子ハプトグロビン遺伝子欠失対立遺伝子（Hp^{del}）の、臨床現場でも利用可能な迅速診断法を確立し、より安全な輸血医療の遂行を目的とする。目的達成のためには迅速化、経費の軽減化、遺伝子解析専門のスタッフを必要としない等のメリットのある簡便な解析法の開発が必要であるが、久留米大学病院臨床検査部輸血検査室は平成21年1月よりHp^{del}診断のための輸血前患者の血液試料の供与を開始した。

A．研究目的

輸血や血液製剤の投与は重篤な副作用の発生頻度の低い比較的安全な医療行為であるとされている。しかしながらウイルス等の感染性副作用についてはその対策が重要である。感染性副作用以外では免疫学的副作用が知られており、さらに免疫学的副作用は溶血性と非溶血性に区分され、現在非溶血性輸血副作用が最も高頻度である。非溶血性輸血副作用は、蕁麻疹や発熱などの軽症例が大部分をしめるが、重篤なアナフィラキシー反応，輸血関連急性肺障害（TRALI）や血液製剤の細菌汚染による死亡例も報告されている。したがって重篤な非溶血性輸血副作用であるアナフィラキシーショックの原因の究明と対策は急務である。アナフィラキシーショックの原因の1つとして血漿タンパク欠損が知られている。そのうち西洋人ではIgA欠損が主要な原因であるが、日本人ではその頻度は低く、ハプトグロビン欠損症の方が高頻度であることから、2005年から各種血液製剤添付資料

「慎重投与の項」に記載されている。

本研究の目的は、より安全な輸血医療の遂行を目的とし、臨床現場でも利用可能な迅速診断法を確立することにより、日本人における血液製剤投与後のアナフィラキーショックの主要な原因遺伝子である *Hp*^{del} の自動解析法の開発である。我々の役割は、研究代表者である神田芳郎久留米大学教授の研究グループによって新たに開発された *Hp*^{del} の自動解析法の評価を目的とした患者の輸血前遺伝子診断のための血液試料の供与と、その検査法が臨床現場で利用可能か否かを評価することである。

B．研究方法

本年度は、我々臨床検査部輸血検査室のスタッフと研究代表者である神田芳郎久留米大学教授の研究グループと研究の進め方、解析の進捗状況、改善点等について電話等により複数回の議論を行った。

まず輸血前診断の開始にあたってどのような形でインフォームドコンセントを得るべきかという問題で議論を重ね、輸血予定患者に行う輸血副作用に関する検査を行う旨の包括的インフォームドコンセントを文章で得るという方法を採用した。

さらにどのような形で血液試料を供給すべきかを議論し、その結果血液を遠心分離した後バッフィーコート分画を供与することで、平成 21 年度 1 月より血液試料の供与を始めた。供与方法は久留米大学病院臨床検査部輸血検査室で取り扱う患者血液が 45 サンプル集まった時点で、バッフィーコート分画約 300 μL を供与するという方法で、試料の匿名化(連結可能匿名化)は久留米大学臨床検査部輸血検査室で行っている。

しかしながらその後バッフィーコート分画約 300 μL では解析に用いる試料の採取(約 1 μL)が困難であることと、実験結果の再現性（リアルタイム PCR の蛍光シグナル強度）にばらつきがあるという理由により、486 番目の試料からは全血約 300 μL の供与に変更している。平成 22 年 3 月 31 日現在で 2862 名の輸血前患者血液を供与している。

（倫理面への配慮）

本研究では輸血前患者血液を供与しているが、輸血前のルーチン検査のために採血は必要でありその血液の一部を用いていること及び供与する血液量が約 300 μL と少量であることから、本研究を実施するための特別な採血は必要としない。またすべての患者に対して輸血副作用に関する検査を行う旨の包括的インフォームドコンセントを文章で得ている。さらに本研究計画の実施は久留米大学倫理委員会の承認を得たうえで行っている。

C．研究結果

供与血液は連結可能匿名化（現在連番を用いている）後に、研究代表者の研究グループに供与している。現時点では輸血副作用が問題となる Hp^{del} のホモ接合体が1名見出されており平成22年3月31日現在名の中に91名の Hp^{del} ヘテロ接合体が検出された。今回見出された1名の Hp^{del} のホモ接合体の患者は術前患者で輸血予定であったが結局輸血は施行されなかった。今後さらに Hp^{del} のホモ接合体が検出された場合は本人に結果を通知し、洗浄赤血球の輸血等の必要な処置を講じてゆく予定である。

D．考察

本研究計画における、当該年度の我々の役割は血液試料の供与であるが、来年度以降は血液試料の供与に加えて、新たに開発された2種類の Hp^{del} の診断法の評価を行ってゆく予定である。現在TaqManプローブを用いた Hp^{del} の接合性を判定するリアルタイムPCR法と、SYBR Green Iを指標としリアルタイムPCR法を用いた Hp^{del} 判定法は既に開発されているが、現在Loop-mediated Isothermal Amplification (LAMP)法やDNAマイクロチップ電気泳動装置を用いた解析法が開発途中であることから、これら複数のシステムのうちどの方法が最も臨床現場での検査方法として適しているのか評価してゆく予定である。さらに本研究計画によって Hp^{del} のホモ接合体であることが判明した患者に対する説明における担当医、検査部の役割、方法について標準化を図りたいと考えている。

E．結論

平成21年1月より Hp^{del} 診断のための輸血前患者の血液試料の供与を開始した。来年度以降は血液試料の供与に加えて、開発された Hp^{del} の診断法の評価を行ってゆく予定である。

F．健康危険情報

なし。

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
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		In press	2010

Ⅳ．研究成果の刊行物・別刷

Nakamura H, Soejima M, Munkhtulga L, Iwamoto S, Koda Y. Haptoglobin polymorphism in Mongolian population: comparison of the two genotyping methods. Clin Chim Acta. 2009 Oct;408(1-2):110-113.

Clinica Chimica Acta 408 (2009) 110–113

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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^1 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^1/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^2	2	1.02	0.98–1.06
HP^2/HP^2	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^3) 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^2/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^3 allele has 3 tandem repeats of the same 1.7-kb DNA segment duplicated in the HP^2 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^2/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^2 and 1 HP^2/HP^3) 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^2 and 1 HP^2/HP^3) were excluded from the analyses.

1.36 in one HP^2/HP^5. 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^3 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^1 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 0.00018 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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ORIGINAL RESEARCH

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*).[6] 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.[16] 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

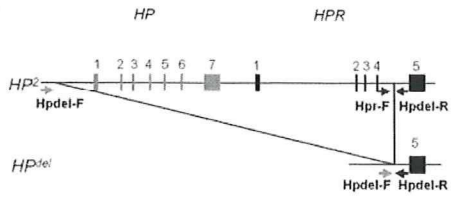

Fig. 1. Structures of the HP^2, and HP^{del} alleles and relative positions of the Hpdel-F, Hpr-F, and Hpdel-R primers.

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 $HP^{\Delta del}$ 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 $HP^{\Delta del}$ and HPR

We investigated duplex real-time PCR, which permits discrimination of $HP^{\Delta del}$ 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 $HP^{\Delta del}$, 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 $HP^{\Delta del}$) 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 $HP^{\Delta del}$ 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 $HP^{\Delta del}$ (*HP/HP*), heterozygous for $HP^{\Delta del}$ (*HP/HP*$^{\Delta del}$), and homozygous for $HP^{\Delta del}$ ($HP^{\Delta del}/HP^{\Delta del}$) were used as templates. We failed to establish an applicable duplex PCR method with similar peak intensities for $HP^{\Delta del}$ 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 $HP^{\Delta del}$ 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 $HP^{\Delta del}$ 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 $HP^{\Delta del}$ product and *HPR* were observed in the genomic DNA sample from *HP/HP*$^{\Delta del}$ individuals, while a single peak (T_m = 80.3-80.7°C) corresponding to the $HP^{\Delta del}$ was observed in genomic DNA sample from the $HP^{\Delta del}/HP^{\Delta del}$ 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 $HP^{\Delta del}$ 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

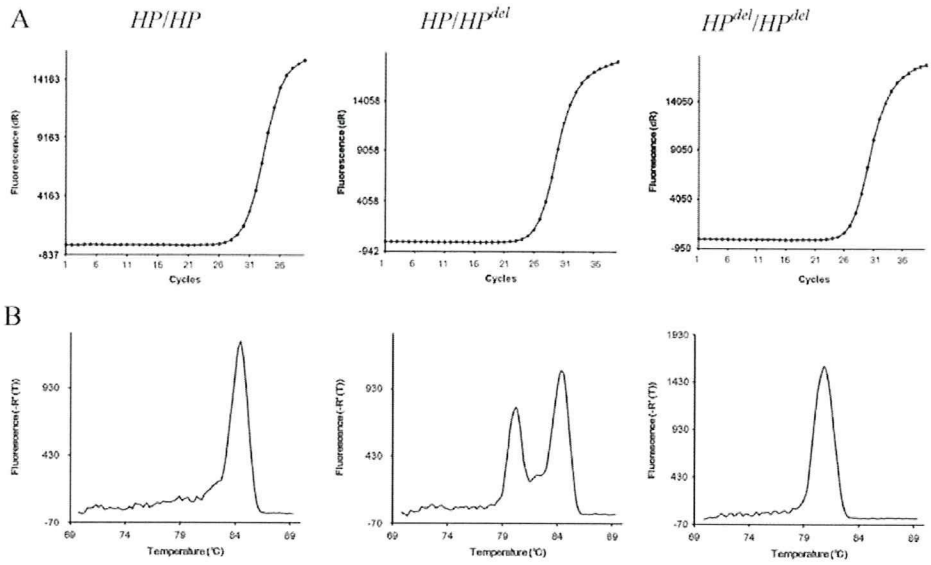

Fig. 2. SYBR Green I–based real-time PCR analysis for detection of HP^{del} and *HP*. Genomic DNA whose genotypes are *HP/HP* (left), HP/HP^{del} (middle), and HP^{del}/HP^{del} (right) were used as templates. (A) Duplex real-time amplification plot for *HPR* (intron 4) and HP^{del} as targets. (B) Dissociation 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

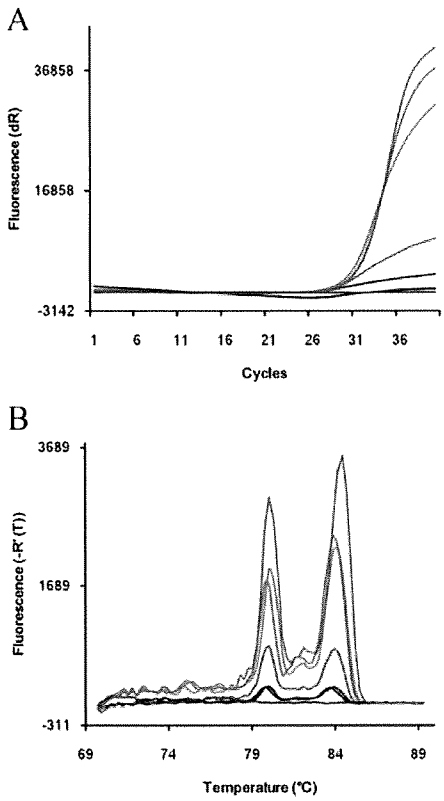

Fig. 3. Effect of dilution of blood on the fluorescent signal of real-time PCR analysis. The results of severalfold diluted blood sample from an $HP1/HP^{del}$ individual are shown by navy (no dilution), red (1:4), green (1:16), gray (1:64), chartreuse (1:256), blue (1:1024), and purple (1:4096). A negative control is shown by orange. (A) Real-time amplification plot. (B) Dissociation curves provide a graphic representation of the PCR product after amplification.

50 mmol/L NaOH, the T_m value seemed to have dropped slightly (approx. 0.5°C for both products) compared to that observed when water is used as the diluent. Although undesired fluorescent signals were detected in some negative control samples (with a frequency of less than 10%), they were not reproducible, and the T_m value (76.8°C) of the undesired products was lower than that of the targets.

For safe transfusion medicine for as many people as possible, the screening test of HP^{del} of every potential transfusion recipient before transfusion is desirable. However, as mentioned above, the prevalence of absolute Hp deficiency is relatively low, and it is a difficult issue to include the screening test considering the cost-effectiveness, while they would only need to be tested once in their lifetime.

The advantages of the TaqMan-based method over the SYBR Green I–based are superior specificity and the possibility of determination of common Hp genotypes (HP^1 and HP^2) when one probe and primers set is added.[21] However, when confirmation of the length of PCR products by electrophoresis was included, the reliability of the SYBR Green I–based method with melting curve analysis was comparable with that of the TaqMan-based method. In addition, the results of the present SYBR Green I–based method with melting curve analysis were completely concordant with that of the TaqMan-based method. When melting curve analysis is included, less than 1.5 hours after taking blood from the subject is required for allele determination like the TaqMan-based method. Thus, the present SYBR Green I–based method for diagnosis of the HP^{del} allele seems to be comparable to the TaqMan-based method in specificity and suitability for high-throughput analysis. It seems to be better adapted to clinical diagnosis of patients before blood transfusion or infusion of human blood components in clinical laboratories as an alternative method for allelic discrimination of HP^{del} to prevent anaphylactoid shock caused by anti-Hp.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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