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 ATTTCAGCGA-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).

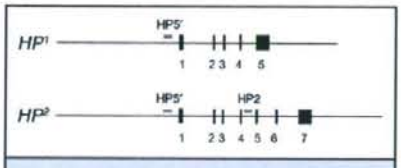

Fig. 1. Structures of the HP^1 and HP^2 alleles and relative positions of the HP2 and HP5′ probes.

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

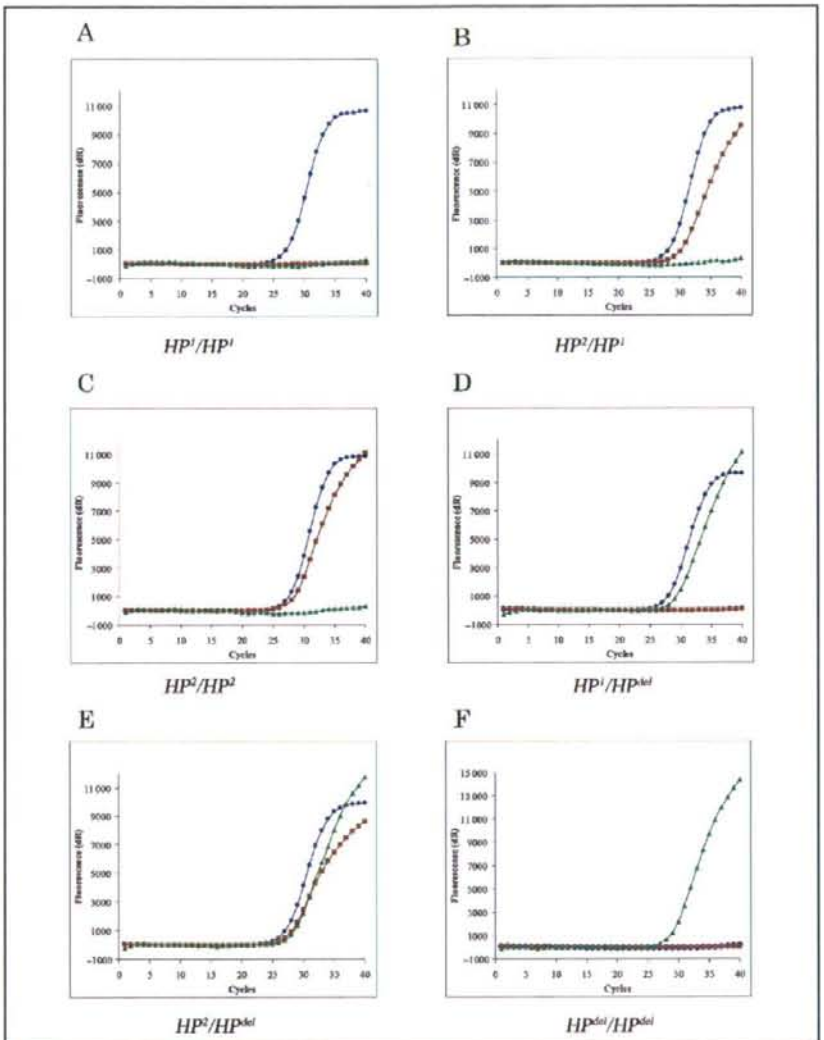

Fig. 2. Real-time amplification plots for HP2, HP5′, and HP^{del} with genomic DNA targets of various genotypes: HP^1/HP^1 (A), HP^2/HP^1 (B), HP^2/HP^2 (C), HP^1/HP^{del} (D), HP^2/HP^{del} (E), and HP^{del}/HP^{del} (F).

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^1 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.

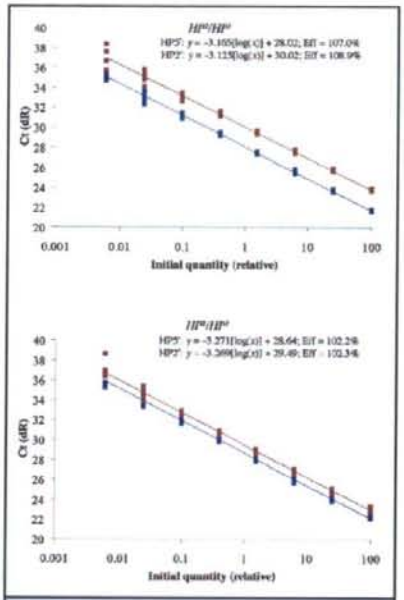

Fig. 3. HP2 and HP5′ calibration curves calculated from Ct data for 6.1 pg to 100 ng DNA template from HP^2/HP^1 and HP^2/HP^2 individuals.

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^1 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^1 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^1. 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^1/HP^1 individuals (n = 105). This absence of an HP^2 signal was defined as indicating an HP^2/HP^1 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^1 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^{del} genotypes (i.e., HP^1/HP^{del}, HP^2/HP^{del}, and HP^{del}/HP^{del}), 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^{del} 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^1-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.

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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)