Clin Genet 2003: 64: 439–443
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CLINICAL GENETICS
ISSN 0009-9163

Short Report

A−61C and C−101G Hp gene promoter polymorphisms are, respectively, associated with ahaptoglobinaemia and hypohaptoglobinaemia in Ghana

Teye K, Quaye I K E, Koda Y, Soejima M, Tsuneoka M, Pang H, Ekem I, Amoah A G B, Adjei A, Kimura H. A−61C and C−101G Hp gene promoter polymorphisms are, respectively, associated with ahaptoglobinaemia and hypohaptoglobinaemia in Ghana.
Clin Genet 2003: 64: 439–443. © Blackwell Munksgaard, 2003

We have investigated the genetic basis for the Hp0 phenotype amongst 123 randomly selected Ghanaians. A total of 17 individuals were determined to be Hp0 phenotype, based on the classical method for Hp phenotyping of Hb-supplemented plasma. Out of the 17 Hp0 individuals, nine subjects were further classified as ahaptoglobinaemic and eight as hypohaptoglobinaemic by Western blots and double immunodiffusion. We identified three previously known base substitutions (A−55G, A−61C and T−104A) and three new ones (C−101G, T−191G and C−242T) within the 5′ flanking region of the *Hp* gene. The A−61C base substitution significantly decreased transcriptional activity and was associated strongly with Hp^2 allele and ahaptoglobinaemia. The C−101G substitution was similar in transcriptional activity to the wild-type and was associated with Hp^{1S} allele and hypohaptoglobinaemia. The Hp^{del} allele seen in Asian populations was absent. We conclude that the Hp0 phenotype in Ghana has a genetic basis that differs significantly from that seen in Asia.

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Key words: ahaptoglobinaemia – Ghanaians – haptoglobin – Hp0 phenotype – hypohaptoglobinaemia – promoter polymorphism

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Received 23 April 2003, revised and accepted for publication 2 July 2003

Haptoglobin (Hp) is an acute phase protein found in all vertebrates. It binds haemoglobin (Hb) to prevent both iron loss and kidney damage during haemolysis. Humans are unique in having a genetic polymorphism of the protein due to two codominant alleles, Hp^1 and Hp^2 which give rise to the three, Hp^1/Hp^1, Hp^2/Hp^1 and Hp^2/Hp^2 genotypes and the Hp1, Hp2–1 and Hp2 resulting phenotypes. Hp^1 has two variants, Hp^{1F} (fast) and Hp^{1S} (slow), as a result of differential migration of their products on starch gels (1, 2). The Hp^2 allele is supposed to originate from a chromosomal aberration (unequal crossing-over) in an individual who was heterozygous for Hp^{1F} and Hp^{1S} (3). A fourth phenotype, Hp0 has only been adequately characterized in Asian populations. In Japan, Korea and China, Hp0 occurs by a deletion (approximately 28 kb) of a segment of chromosome 16 extending from the promoter region of the *Hp* gene to exon 5 of the haptoglobin-related gene (*Hpr*) (4, 5). The corresponding allele is named Hp^{del}. The Hp0 phenotype of these individuals results from the Hp^{del}/Hp^{del} homozygous genotype.

The Hp0 phenotype is prevalent (10–40%) in Sub-Saharan Africa (6), but unlike the population in Asia, the precise cause of Hp0 in Africans is currently unknown. Serum Hp levels decrease following haemolysis, as Hp clears released haemoglobin. In malaria infection, such benign

haemolytic episodes occur accounting partially for reduced Hp levels in individuals within the population (7–10). The Hp^{del} allele is the only characterized allele for the Hp0 phenotype, but it has not yet been seen in any other population (5). Ahaptoglobinaemia has important clinical consequences. In East Asians, it is responsible for anaphylactic reactions in blood transfusions (5, 11). In Africa, Hp0 has also been associated with improved HIV prognosis (12).

In the present paper, we have examined the nature of the Hp0 phenotype in general and *Hp* gene promoter polymorphism specifically, as contributing to the Hp0 phenotype in Ghana, a malaria-endemic country in West Africa.

Materials and methods

Blood sample collection and isolation of genomic DNA

Venous blood was collected from 123 randomly selected donors into ethylenediamine tetraacetic acid tubes between May and June of 2001, at the Korle-Bu Teaching Hospital of the University of Ghana Medical School, Ghana. Thick and thin blood films were made to exclude malaria from the donors. Both plasma and buffy coat were obtained after centrifugation. Genomic DNA was isolated from frozen buffy coat using a QIAamp DNA kit (Qiagen, Chatsworth, CA). The project was approved by the Ethical and Review Committee of the University of Ghana Medical School, Ghana.

Haptoglobin phenotyping

Hp phenotypes were determined by discontinuous polyacrylamide gel (7.5%) electrophoresis (PAGE) of Hb-supplemented plasma, followed by staining with *o*-dianisidine (Sigma-Aldrich Fine Chemicals, St. Louis, MO) and 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma-Aldrich). After electrophoresis, the gel was fixed in 10% trichloroacetic acid for 5 min before staining with 0.05% *o*-dianisidine and 0.3 mM TMB in 1.75 M sodium acetate buffer, pH 5.0, containing 20% methanol. The stain was activated with 0.05% hydrogen peroxide.

Characterization of ahaptoglobinaemia and hypohaptoglobinaemia

Samples that showed no bands after staining of Hb–Hp complex were probed for the presence of Hp by double immunodiffusion and Western blotting, using goat antibody to human Hp (Dako Japan, Tokyo, Japan). Samples that were still negative for Hp by double immunodiffusion and Western blotting were characterized as ahaptoglobinaemic and those that were positive as hypohaptoglobinaemic.

PCR and genotype determination

Polymerase chain reaction (PCR) amplification was performed to detect Hp^{del} allele, using the specific primers Hp-del-U and Hp-del-L, as described previously (5). *Hp* genotypes were determined by PCR, using Hp-Exon-1 U and Hp-Exon-7 L primers (5). Hp^{1} variants were also examined by amplifying exons 2–4, using Hp-Exon-2 U and Hp-Exon-4 L primers (5). Restriction digests with *Xba*I of the PCR product enabled a differentiation of the Hp^{1S} and Hp^{1F} alleles (13). The promoter region was examined by amplification of a 477-bp fragment of the 5′ flanking region with Hp-Exon-1 U and Hp-Exon-1 L primers, as described previously (4). PCR products were purified and sequenced directly with an ABI PRISM dye terminator cycle-sequencing ready reaction kit (Applied Biosystems, Tokyo, Japan).

Construction of luciferase reporter gene and assessment of Hp promoter activity

We observed novel mutations within the promoter region of the Hp gene in addition to mutations that had previously been reported. To determine whether these mutations had an effect on *Hp* gene expression, we amplified the regions harboring the mutation with primers designed to have *Hin*dIII and *Xba*I restriction sites. The products were subcloned into pGEM-T vector (Promega, Madison, WI) and sequenced. After confirming that the clones harbored the mutations (−55G −104A, −61C, −101G and −191G−242T), *Hin*dIII and *Xba*I digests were performed to release a 647-bp fragment. This was cloned into *Hin*dIII and an *Nhe*I compatible site on the promoterless luciferase vector pGL3-basic (Promega). One microgram of each of the purified plasmid was transfected into HepG2 cells, using a FuGene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). After 48 h, the cells were lysed, and firefly luciferase activity was measured using a dual-luciferase reporter assay system (Promega) and a luminometer (Lumat LB9501, Berthhold, Wildbad, Germany). Transfection efficiency was normalized by cotransfection with the pRL/CMV vector and measurement of *Renilla reiformis* luciferase activity. The promoter activity was determined by the fold of increase of light units

relative to that obtained from transfection of the promoterless pGL3-basic vector. Assays were repeated three times, and the cells were not stimulated before luciferase activity was measured.

Haplotype determination

Allele frequencies were determined by gene counting. Maximum likelihood haplotypes were estimated, based on an Expectation-Maximization (EM) algorithm using ARLEQUIN software (14).

Statistics

Differences between Hp groups were compared by means of the Fisher's exact test and those between promoter activities by student's *t*-test. A *P*-value <0.05 was considered significant.

Results

Occurrence of Hp0 phenotype in Ghanaians

We determined that 17 (13.8%) of the 123 Ghanaian subjects with undetectable malaria parasite infection were Hp0, based on the classical method for Hp phenotyping of Hb-supplemented plasma. However, by double immunodiffusion followed by Western blotting, we have categorized these individuals as either ahaptoglobinaemic or hypohaptoglobinaemic. There were nine ahaptoglobinaemic (7.3%) and eight hypohaptoglobinaemic (6.5%) individuals (Table 1).

Table 1. Genotypes of ahaptoglobinaemic and hypohaptoglobinaemic subjects

Sample number	Sex	Genotype
Ahaptoglobinaemia ($n=9$)		
1	M	$Hp^2/-55G-104AHp^{1F}$
2	F	$-61CHp^2/-61CHp^2$
3	F	Hp^2/Hp^2
4	M	$-61CHp^2/-55G-104AHp^{1F}$
5	F	$-61CHp^2/-55G-104AHp^2$
6	F	$Hp^2/-61CHp^2$
7	F	$Hp^2/-55G-104AHp^{1F}$
8	M	$-61CHp^2/-55G-104AHp^{1F}$
9	M	$Hp^2/-55G-104AHp^{1F}$
Hypohaptoglobinaemia ($n=8$)		
1	F	$-101GHp^{1S}/-55G-104AHp^{1F}$
2	M	$-101GHp^{1S}/-101GHp^{1S}$
3	M	$-101GHp^{1S}/-101GHp^{1S}$
4	M	$-61CHp^2/-101GHp^{1S}$
5	F	$Hp^2/-55G-104AHp^{1F}$
6	M	Hp^2/Hp^2
7	M	$Hp^2/-61CHp^2$
8	M	$-61CHp^2/-55G-104AHp^{1F}$

The genotypes in Hp0 individuals

Of the nine ahaptoglobinaemic subjects, four were Hp^2/Hp^2 and five were Hp^2/Hp^{1F}, while the 8 hypohaptoglobinaemic subjects consisted of two individuals with Hp^{1F}/Hp^{1S}, two Hp^{1S}/Hp^{1S}, one Hp^2/Hp^{1F}, one Hp^2/Hp^{1S} and two individuals with Hp^2/Hp^2. We found Hp^2 in 13 (72%) out of 18 alleles of ahaptoglobinaemic individuals, suggesting a marginal association of the Hp^2 allele with ahaptoglobinaemia (Table 2) ($P=0.0489$ against the control group), whereas the frequency of the Hp^2 allele (44%) in hypohaptoglobinaemic subjects was similar to that of the control group (Table 2). None of the ahaptoglobinaemic individuals had an Hp^1/Hp^1 genotype, even though the distribution of Hp^1/Hp^1 and Hp^2/Hp^2 were similar in the study population, as revealed by the similarity between the allele frequency distribution of Hp^1 and Hp^2 (Table 2). In contrast, Hp^1/Hp^1 was present in the hypohaptoglobinaemic group and predominantly as Hp^{1S} (Table 2) ($P=0.0411$). We did not find the *Hp* gene deletion (Hp^{del}) in Ghanaians.

Hp promoter sequences: association of −61C allele with ahaptoglobinaemia and of −101G with hypohaptoglobinaemia

In addition to three previously known base substitutions (A−55G, A−61C and T−104A), we also found three new ones (C−101G, T−191G and C−242T) in the 5′ flanking region of the *Hp* gene of Ghanaians (Tables 2 and 3). The frequencies of the six base substitutions in the 5′ flanking region are summarized in Table 2.

We found an association between the −61C allele and ahaptoglobinaemia. Four heterozygotes and one homozygote out of the nine ahaptoglobinaemic individuals carried the base substitution (Table 2) ($P=0.0125$ against the control group). We also observed that the new C−101G base substitution found in this study was strongly associated with hypohaptoglobinaemia (Table 2) ($P=0.0187$).

Maximum likelihood haplotypes of the base substitutions in the 5′ flanking region were estimated by ARLEQUIN software (14) and summarized in Tables 1 and 3. We found that the −61C and −101G mutations were strongly in linkage disequilibrium with the Hp^2 and Hp^{1S} alleles, respectively (Table 3). The A−55G and T−104A substitutions are almost always associated with Hp^{1F} allele in the population (Table 3). The −191G and −242T occurred with very low frequencies.

Table 2. Single nucleotide polymorphism (SNP) and haptoglobin (Hp) allele and frequencies amongst controls, hypohaptoglobinaemic and ahaptoglobinaemic subjects

	Total ($n=123$)	Control ($n=106$)	Hypohaptoglobinaemia ($n=8$)	Ahaptoglobinaemia ($n=9$)
Allele (with their frequencies)				
Hp^{1F}	0.354 (87)[a]	0.373 (79)	0.188 (3)	0.278 (5)
Hp^{1S}	0.163 (40)	0.160 (34)	0.375 (6) ($P=0.0411$)	0 (0)
Hp^{2}	0.484 (119)	0.467 (99)	0.438 (7)	0.722 (13) ($P=0.0489$)
Hp^{del}	0 (0)	0 (0)	0 (0)	0 (0)
SNP (with their frequencies)				
−55G	0.419 (103)	0.434 (92)	0.188 (3) NS	0.333 (6) NS
−61C	0.126 (31)	0.104 (22)	0.188 (3) NS	0.333 (6) ($P=0.0125$)
−101G	0.138 (34)	0.132 (28)	0.375 (6) ($P=0.0187$)	0 (0)
−104A	0.374 (92)	0.387 (82)	0.188 (3) NS	0.333 (6) NS
−191G	0.004 (1)	0.005 (1)	0 (0)	0 (0)
−242T	0.012 (3)	0.014 (3)	0 (0)	0 (0)
Sex (M/F)	44/79	34/72	6/2	4/5
Mean age ± SD	28.3 ± 15.9	29.3 ± 16.4	19.4 ± 9.7	24.0 ± 11.6

NS, not significant.
[a]Number of occurrence of the alleles and SNPs.

Promoter activities associated with base substitutions in the 5′ flanking region of the Hp gene

A graph of the relative promoter activity associated with base substitutions in the 5′ flanking region using luciferase reporter gene in human HepG2 cells is shown in Fig. 1. The −61C allele is associated with a significantly decreased promoter activity ($P=0.02$), as reported previously (15). The promoter activities of DNA fragments containing C−101G, A−55G, T−104A, T−191G and C−242T base substitutions did not lead to any significant changes in promoter activity.

Discussion

We estimate that the proportion of the Hp0 phenotype is 13.8% in the Ghanaian population, based on the classical method of phenotyping of Hb-supplemented plasma. We have further shown that the 'Hp0' phenotype can be subdivided into ahaptoglobinaemic and hypohaptoglobinaemic groups, based on immunological methods. In this paper, we have used 'Hp0' phenotype as a general term for describing the two groups combined, in the Ghanaian population.

In East Asians, the ahaptoglobinaemia has previously been characterized in people homozygous

Table 3. Haptoglobin (Hp) haplotype frequencies within the Ghanaian population

Allele	Frequency (123)[a]
Hp^{1F}	0.004
$-55GHp^{1F}$	0.013
$-55G-104AHp^{1F}$	0.337
Hp^{1S}	0.014
$-61CHp^{1S}$	0.006
$-61C-191G-242THp^{1S}$	0.004
$-101GHp^{1S}$	0.138
Hp^{2}	0.291
$-55GHp^{2}$	0.036
$-55G-104AHp^{2}$	0.033
$-61CHp^{2}$	0.116
$-242THp^{2}$	0.008
Hp^{del}	0

[a]Number of subjects examined.

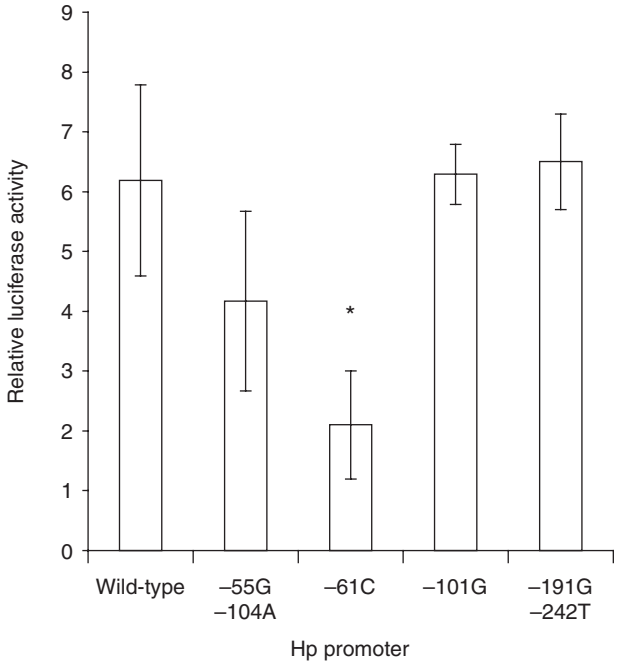

Fig. 1. Promoter activities associated with base substitution of the 5′ flanking region of the haptoglobin (*Hp*) alleles identified in the Hp0 individuals. Plasmids containing various base substitutions ligated to luciferase vector were transfected into HepG2 cells, and luciferase activity in cells was measured after 48 h. Error bars indicate standard deviations for three experiments. $*P=0.02$. The wild-type is the reference construct.

for the Hp^{del} allele, whereas people heterozygous for the Hp^{2}/Hp^{del} were shown to be hypohaptoglobinaemic (4, 5). We did not find this allele in the Ghanaian population that we examined, suggesting that the cause of 'Hp0' phenotype in Ghana appears to be more complicated than in Asian populations. Further investigations would consider whether this is the general picture in Ghana and other parts of Africa. We found for the first time that the A−61C base substitution and Hp^{2} allele significantly associate with ahaptoglobinaemia (Table 2). We have also seen that the new base substitution C−101G, found in the Ghanaian population on the Hp^{1S} allele, was strongly associated with hypohaptoglobinaemia. The A−61C mutation has previously been associated with the development of the Hp2–1 mod phenotype (13). It was seen to significantly reduce promoter activity (15), as was observed in the present study. The mutation also reduces the responsiveness of *Hp* gene to interleukin-6 (IL-6) in human hepatoma cells (15), as the −61 position lies in one of three IL-6-responsive elements for the transactivation of the *Hp* gene (15–17). It is important for us to point out that not all individuals with the mutation were Hp0 and not all Hp0 individuals carried the mutation. This means that in addition to the A−61C mutation, other factors contribute to the Hp0 phenotype. Nevertheless, individuals with the A−61C mutation would likely appear phenotypically Hp0, when stress conditions like haemolysis are augmented. We observed that the transcriptional activity for the C−101G base substitution is similar to the wild-type. This mutation may be a major reason for hypohaptoglobinaemia rather than ahaptoglobinaemia. In general, it is postulated that during malaria disease, benign haemolysis occurs from both infected and uninfected red blood cells that leads to Hp depletion, as it mops up released haemoglobin (6, 8–10). Our observed association between the −61C and −101G alleles with a- and hypo-haptoglobinaemia, respectively, indicates that a genetic basis of Hp0 exists in Ghana. Future work will aim to screen a larger population to determine whether any other genetic causes for the Hp0 phenotype exist in Africa.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, the Novartis Foundation (Japan) and the Mitsui Life Social Welfare Foundation.

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