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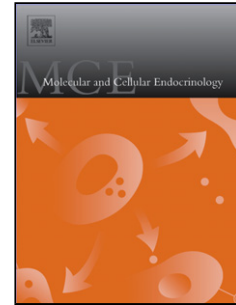
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## **Growth Hormone (*GHI*) Gene Variation and the Growth Hormone Receptor (*GHR*) Exon 3 Deletion Polymorphism in a West-African Population**

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**Abstract**

Among Europeans, functionally significant *GHI* gene variants occur not only in individuals with idiopathic growth hormone (GH) deficiency and/or short stature but also fairly frequently in the general population. To assess the generality of these findings, 163 individuals from Benin, West Africa were screened for mutations and polymorphisms in their *GHI* genes. A total of 37 different sequence variants were identified in the *GHI* gene region, 24 of which occurred with a frequency of >1%. Although four of these variants were novel missense substitutions (Ala13Val, Arg19His, Phe25Tyr and Ser95Arg), none of these had any measurable effect on either GH function or secretion *in vitro*. Some 37 different *GHI* promoter haplotypes were identified, 23 of which are as yet unreported in Europeans. The mean *in vitro* expression level of the *GHI* promoter haplotypes observed in the African population was significantly higher than that previously measured in Britons ( $p < 0.001$ ). A gene conversion in the *GHI* promoter, previously reported in a single individual of British origin, was found to occur at polymorphic frequency (5%) in the West-African population and was associated with a 1.7-fold increase in promoter activity relative to the wild-type. The d3 allele of the *GHR* exon 3 deletion polymorphism, known to be associated with increased GH responsiveness, was also found to occur at an elevated frequency in these individuals from Benin. We speculate that both elevated *GHI* gene expression and increased GHR-mediated GH responsiveness may constitute adaptive responses to the effects of scarce food supply in this West-African population since increased circulating GH appears to form part of a physiological response to nutritional deprivation.

**Keywords:** Growth hormone; Growth hormone receptor; Benin; Missense mutations; Promoter haplotype; Gene conversion; Gene expression

## 1. Introduction

Human growth hormone (GH) is a pleiotropic cytokine that promotes the postnatal growth of skeletal and soft tissue as well as acting as an important regulator of bone turnover, muscle mass and immune function. The biological action of GH is mediated through the activation of its cell surface receptor, GHR. Each GH molecule has two binding sites, ensuring that the binding of GH to GHR results in the formation of a receptor homodimer. Interaction of dimerized GHR with the intracellular tyrosine kinase JAK-2 leads to the phosphorylation of downstream signal transduction molecules, induction of signal transducers and activators of transcription (STAT) proteins, and stimulation of mitogen-activated protein (MAP) kinases (Waters et al., 2006). Activated STAT5 is then translocated to the nucleus where it transactivates a series of GH-responsive genes.

In humans, GH synthesis is directed by the pituitary-expressed *GHI* gene which is located on chromosome 17q23 within a cluster of five highly homologous and placentally expressed genes: two chorionic somatomammotropin genes (*CSH1* and *CSH2*), a second GH-related gene (*GH2*) and a chorionic somatomammotropin pseudogene (*CSHP1*). *GHI* gene expression is controlled both by the proximal promoter and a locus control region (LCR) located between 14.5kb and 32kb upstream of the *GHI* gene (Jones et al., 1995). The LCR contains multiple DNase I hypersensitive sites and is required for the activation of the genes of the GH cluster in both pituitary and placenta (Su et al., 2000; Ho et al., 2002). Two DNase I hypersensitive sites (I and II) contain binding sites for the pituitary-specific transcription factor Pit-1 and are responsible for the high level-, somatotroph-specific expression of the *GHI* gene (Shewchuk et al., 1999, 2002). It has been shown that polymorphic variation in the proximal promoter region of the *GHI* gene influences *GHI* gene expression and may thus contribute to the heritability of human stature (Hasegawa et al., 2000; Horan et al., 2003; Giordano et al., 2006; Giordano et al., 2008). The proximal promoter region of the *GHI* gene contains at least 15 single nucleotide polymorphisms (SNPs) (Giordano et al., 1997; Wagner et al., 1997). In the European population, this variation has been found to manifest itself in at least 40 different haplotypes, the high diversity being explicable in terms of gene conversion, recurrent mutation and selection (Giordano et al., 1997; Horan et al., 2003; Chen et al., 2007; Wolf et al., 2008). Functional analysis demonstrated that haplotypes associated with a markedly reduced level of reporter gene expression were more prevalent in the European population than those haplotypes associated with an increased level, possibly as a consequence of selection (Horan et al., 2003). It has also been demonstrated that *GHI* gene variants occur not only in individuals with idiopathic GH deficiency and/or short stature but also fairly frequently in the general (European) population (Millar et al., 2003; Esteban et al., 2007).

The human growth hormone receptor (*GHR*) gene on chromosome 5p13-p12 is also polymorphic, with polymorphisms described in exons 3, 6 and 10 (Buzi et al., 2007). The exon 3 polymorphism gives rise to exon 3-containing (fl) and exon 3-deleted (d3) isoforms (Pantel et al., 2000). In the Western European population, the d3 (deletion) allele occurs at a frequency of ~50% (Dos Santos et al., 2004; Binder et al., 2006; Pilotta et al., 2006; Carrascosa et al., 2006; Blum et al., 2006) and has been reported to be associated with an increased growth response to exogenous human GH (Dos Santos et al., 2004). This has, however, become a contentious issue since although some studies have concurred with the initial findings (Jorge et al., 2006; Binder et al., 2006; Jensen et al., 2007; Tauber et al., 2007; Binder et al., 2008; Ráz et al., 2008),

various other studies (Pilotta et al., 2006; Carrascosa et al., 2006; Blum et al., 2006; Kenth et al., 2007; Carrascosa et al., 2008) have failed to replicate the original association (Buzi et al., 2007).

In an attempt to test the generality of the above mentioned observations in a non-European population, a group of healthy individuals from Benin, West Africa was screened for mutations and polymorphisms in their *GHI* genes and genotyped for the *GHR* exon fl/d3 deletion polymorphism.

## 2. Materials and Methods

### 2.1. DNA samples

DNA samples were obtained from 163 individuals from Benin, West Africa, as previously described (Pepe et al., 1999). These individuals came from four different tribes: Bariba (41 individuals; 30 males, 11 females), Berba (33 individuals; 13 males, 20 females), Fon (44 individuals; 29 males, 15 females) and Dendi (45 individuals; 34 males, 11 females). No height data were available from these individuals. DNA samples from 156 male British army recruits (Horan et al., 2003; Millar et al., 2003) were also included in the analysis of the *GHR* d3 polymorphism.

### 2.2. Detection of sequence variants within the *GHI* gene

A 3.2 kb fragment specific to the *GHI* gene was PCR-amplified for all individuals (Millar et al., 2003) using oligonucleotide primers GH1F (5' GGGAGCCCCAGCAATGC 3'; -615 to -599) and GH1R (5' TGTAGGAAGTCTGGGGTGC 3'; 2598 to 2616) [numbering relative to the transcriptional initiation site at +1 (GenBank accession number J03071)]. These PCR fragments were then directly sequenced to identify sequence variants within the entire coding region, introns, 5' and 3' untranslated regions and promoter of the *GHI* gene. The oligonucleotide primers used for sequencing were GH1BF (5' GTGGTCAGTGTGGAAGTGC 3'; -556 to -537), GH1SEQ1 (5' CCACTCAGGGTCCTGTG 3'; +27 to +43), GH1SEQ2 (5' GGAGGAGACTAAGGAGCTC 3'; +556 to +584) and GH1SEQ3 (5' TTAGAGAAACACTGCTGCCC 3'; +1137 to +1156) as previously described (Millar et al., 2003). In cases where individuals were heterozygous at two or more positions throughout the gene, the 3.2 kb *GHI* gene fragments were cloned into the cloning vector pGEM-T (Promega, UK) and four clones were sequenced to identify unambiguously the respective haplotypes.

### 2.3. Haplotype analysis of the exon 3 *GHR* polymorphism

Haplotype analysis of the exon 3 *GHR* polymorphism was carried out in 156 Britons and 154 Beninese using quantitative (Q-PCR) PCR as previously described (Horan et al., 2006). Briefly, primer/probe sets targeted to exons 3 and 10 of the human *GHR* gene. *GHR* exon 10 served as an internal positive control. Oligonucleotide primers and probe used for exon 3 of the *GHR* gene were: GHRX3F (5' TCTGTTTCAGCCACAGCAGCTA 3'; +159 to +180) and GHRX3R (5' TGTCTTTAGGCCTGGATTAACACTT 3'; +233 to 209) with the FAM-labelled probe GHRX3 (5' FAM-CCTTAGCAGAGCACCCCTGGAGTCTGC-TAMRA 3'; +182 to +207). Oligonucleotide primers and probe used for exon 10 were: GHRX10F

(5' CCCAGGTGAGCGACATTACA 3'; +2471 to +2490) and GHRX10R (5' CATCCCTGCC TTATTCTTTTGG 3'; +2541 to +2519) with the VIC-labelled probe GHRX10 (5' VIC-CAGCAGGTAGTGTGGTCC TTTCCCCG-TAMRA 3'; +2492 to +2517). Primer and probe positions are based on GenBank accession number AH002706. Q-PCR s performed in 96 well plates with 50ng genomic DNA in a volume of 12.5µl using 2x Taqman Universal PCR Master Mix (Applied Biosystems), 1.25µl *GHR* exon 3 primers/probe (3pm/µl each), 1.25µl *GHR* exon 10 primers/probe (9pm/µl each) and 2.75µl sterile water. Cycle conditions were 50°C 2 min, 95°C 10 min followed by 40 cycles of 95°C 15 sec, and 60°C 1 min.

#### 2.4. Analysis of promoter haplotypes by luciferase reporter gene assay

Individual examples of the 26 different *GHI* proximal promoter haplotypes that were both novel and specific to the Beninese population (Table 2) were PCR-amplified using oligonucleotide primers GHPROM5 (5' AGATCTGACCCAGGAGTCCTCAGC 3'; -520 to -501) and either GHPROM3A (5' AAGCTTGCAGCTAGGTGAGCTGTC 3'; + 44 to +62) or GHPROM3C (5' AAGCTTGCCCGCTAGGTGAGCTGTC 3'; + 44 to +62) depending upon the base at position +59 of the haplotype. To facilitate cloning, partial or complete restriction endonuclease recognition sites were added to the 5' end of the primers (underlined above): *Bgl*III (GHPROM5) and *Hind*III (GHPROM3A and GHPROM3C). Following restriction endonuclease digestion, fragments were cloned into the luciferase reporter gene expression vector pGL3Basic (Promega, UK) as described by Horan et al. (2003).

Luciferase reporter gene assays were then performed in rat GC pituitary cells in sextuplicate on three separate occasions as described (Horan et al., 2003). Briefly, GC cells were plated out into 96-well plates so as to be ~80% confluent the following day in DMEM containing 15% horse serum and 2.5% fetal calf serum (FCS). The transfection mixture contained 250ng pGL3 haplotype construct, 1ng pRL-CMV (Promega) and 0.5µl Tfx™-20 (Promega) in a total volume of 90µl serum-free medium per well. After 1 hr, 200µl complete medium was added to each well and incubated for 24 hr at 37°C in 5% CO<sub>2</sub> before being lysed for the reporter assay. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Assays were performed on a microplate luminometer (Applied Biosystems) and then normalised with respect to Renilla activity.

#### 2.5. Site-directed mutagenesis

Site-directed mutagenesis (SDM) was performed as previously described (Millar et al., 2003) on the luciferase reporter gene construct, pGL3H43, containing the *GHI* gene promoter haplotype 43. SDM was used to change the region -53 to -39 of the *GHI* gene promoter sequence to that of the paralogous *CSH1* gene promoter sequence (a change which corresponds to the common gene conversion polymorphism noted in the West-African population). This construct (pGL3H43GC) and pGL3H43 were then used in a second SDM reaction to introduce the associated A+10G sequence variant (which was invariably found in *cis* to the gene conversion) into both constructs to yield pGL3H43GC+10G and pGL3H43+10G respectively. Following SDM, all mutated *GHI* promoter/luciferase reporter gene constructs were sequenced in order to ensure that only the desired changes had been introduced.

#### 2.6. In vitro expression of missense GH variants

Wild-type *GHI* cDNA (transcript variant 1; Accession number NM\_000515) was cloned into the insect expression vector pIZ/V5-His (Invitrogen) and modified by SDM to generate cDNA constructs containing the novel missense GH variants detected in the present study (Millar et al., 2003). These vectors were then transfected into High Five insect cells (Invitrogen) as described by Millar et al. (2003). Human GH was quantified in the culture supernatants by ELISA (DRG Instruments GmbH, Marburg, Germany). The cross-reactivity in the ELISA of the GH variant and insect cell-expressed wild-type GH was confirmed by dilution analysis to be equal to that of the assay reference preparation (calibrated against the MRC 1st IRP 80/505 reference preparation).

#### 2.7. Luciferase reporter gene assay of *STAT 5* activation

HK293 cells, previously transfected with the full-length human GHR gene and selected on the basis of elevated GHR expression (HK293hi) (Ross et al., 1997), were used to assay the *STAT 5* activation potential of each GH-coding sequence variant. HK293hi cells were transfected with a *STAT 5*-responsive luciferase reporter gene construct and treated with GH (wild-type and variant) for 6 hours. Luciferase expression was measured as previously described (Lewis et al., 2004).

#### 2.8. *GH* secretion assays

The rat pituitary (GC) cell line was transfected with a pGEM-T plasmid containing a 3.2 kb fragment spanning the entire wild-type *GHI* gene (under the control of promoter haplotype 1) and equivalent constructs for the missense variants also under the control of haplotype 1. Cells (200,000 cells per well) were plated onto 24-well plates and cultured in complete medium (DMEM, 15% Horse serum, 2.5% FCS). Cells were transfected with 500ng *GHI* plasmid and  $\beta$ -galactosidase expression vector (pCH110; GE Healthcare) using 1 $\mu$ l Tfx<sup>TM</sup>-20 in 200 $\mu$ l serum-free medium for 1 hr after which 0.5ml complete medium was added to each well. Cells were cultured for 48 hr, medium harvested, and cells lysed for  $\beta$ -galactosidase assay to correct for differences in transfection efficiency. GH in the medium was measured for all variants using a human GH IRMA (Nichols Institute Diagnostics) that showed no cross-reactivity to rat GH.

#### 2.9. Molecular modelling

Missense variants were modelled as previously described (Millar et al., 2003).

#### 2.10. Determination of evolutionary conservation of specific *GH* amino acid residues

The evolutionary conservation of the amino acid residues harbouring missense changes was examined by ClustalW multiple sequence alignment [<http://www.ebi.ac.uk/Tools/clustalw>] of orthologous GH proteins from 19 vertebrate species, including mammals, birds, reptiles, amphibians and fish (see Supplementary Table 1 and Supplementary Figure 1).

#### 2.11. Statistical analysis

Luciferase measurements of *GHI* promoter-driven transcription were normalised as follows: First, *GHI* promoter activity values were divided by the corresponding Renilla activity value of the same construct. Next, the mean ratio of the negative controls on a given plate was subtracted from all other ratios on the same plate. The mean (plate-corrected) activity ratio for proximal promoter haplotype 1 (H1) on each plate was then calculated, and all other haplotype-specific activity ratios on the same plate divided by this value. These two transformations ensured that the mean

normalised activity of the negative controls equalled zero whilst the mean normalised activity of H1 equalled unity, independent of plate number. Normalised activities may thus be interpreted as -fold changes by comparison with H1, corrected for transcription efficiency, baseline and plate effects. Since no significant plate effects were detectable after transformation, the data were combined over all plates. The population difference in terms of the mean expression level of haplotypes was assessed for statistical significance using a Student t-test. The relationship between the mean expression levels and the population frequencies of haplotypes was analysed using Spearman rank correlation coefficients, with significance assessment carried out using a z-approximation. Allele frequency differences between populations were tested for statistical significance using a Pearson  $\chi^2$  test. Differences between genotype-specific and wild-type bioactivity measurements ( $EC_{50}$  and percentage GH secretion) were assessed for statistical significance using ANOVA.

### 3. Results

A total of 37 different sequence variants (excluding the 15 promoter SNPs used to define the promoter haplotypes) were identified in the *GHI* genes of 163 Beninese individuals. Of these variants, 13 occurred in the *GHI* gene promoter/5' UTR, 15 within intronic sequence and 9 in the coding sequence. Twenty-four (65%) of the detected variants occurred at a frequency of >1% in our study (7 promoter, 11 intronic, 6 coding sequence) and may therefore be formally regarded as being polymorphisms in the Beninese population. Table 1 lists the frequency and location of these polymorphic *GHI* variants together with the predominant associated promoter haplotype (if any). Among these variants were three novel coding sequence changes (Ala13Val, Phe25Tyr and Ser95Arg) and a gene conversion spanning a minimum of 16 bp of the *GHI* promoter/5'UTR. In addition, solitary examples of Arg19His and Ile179Met substitutions were detected in association with promoter haplotypes H43 and H45, respectively.

In addition, a total of 37 different *GHI* promoter haplotypes were identified in the 163 Beninese individuals studied (Table 2). The observed *GHI* promoter haplotype frequencies in the Beninese samples were compared to those reported from 303 white Europeans (156 British, 116 Spanish, 31 French), previously genotyped by Horan et al. (2003) and Lewis et al. (2004). Of the Beninese promoter haplotypes, 23 were novel whereas 14 had been previously identified in individuals of European origin. The most common promoter haplotype in Africans (H1) was found to be identical to that predominating in Europeans. Although the two next most common promoter haplotypes in the Beninese population (H8 and H23) were also found in Europeans, their frequency in the Beninese was considerably higher. When assessed in a luciferase reporter gene assay (Table 2), the relative promoter strength of the novel Beninese promoter haplotypes was found to vary only two-fold (82% to 170% that of H1), a somewhat lower range than that previously observed among Europeans (33% to 189%; Horan et al., 2003; Table 2). Accordingly, the mean normalised expression level (relative to H1) was significantly higher among African alleles (98.6%, SD: 29.3%) than among European alleles (83.9%, SD: 23.6%, Student t: 8.243, 930 d.f.,  $p < 0.001$ ). On the other hand, whilst the population frequencies of the *GHI* haplotypes were inversely correlated with their respective *in vitro* expression levels (as measured by the mean luciferase activity, and given in Table 2) in the European group (Spearman rank correlation coefficient  $r = -0.361$ ,  $p = 0.02$ ), this was not the case for the Beninese group ( $r = -0.189$ ,  $p = 0.26$ ).

Of the nine coding sequence variants detected in this study, six were associated with missense changes, of which four were novel (Ala13Val, Arg19His, Phe25Tyr and Ser95Arg). Two variants (Thr3Ala and Ile179Met) have been previously described (Miyata et al., 1997; Lewis et al., 2004).

The Thr3Ala variant previously noted in both Europeans (Millar et al., 2003) and Japanese (Miyata et al., 1997) is considered to be a neutral polymorphism lacking any functional effect (Millar et al., 2003). The presence of the Ala3 variant on a variety of different *GHI* promoter haplotypes in three different racial groups is compatible with the occurrence of multiple independent gene conversion events during and after the expansion of modern humans out of Africa. Such gene conversion events could have been mediated by the *CSHP1*, *GH2* or *CSH2* gene.

The Ile179Met missense mutation, previously reported in a Spanish child of short stature, has been found to result in the loss of the ability of GH to activate the extracellular signal-regulated kinase pathway despite having normal ability to activate STAT5 (Lewis et al., 2004). In the Spanish patient, the nucleotide substitution underlying Ile179Met (C+1491G) was present on an H1 haplotype background and was in *cis* to +1169T. By contrast, the solitary example of the occurrence of this mutation in a Beninese individual was associated with haplotype H45 and was in *cis* to +1169T and two other sequence variants, A-360G and T-352G. Since A-360G and T-352G were not associated with the Ile179Met mutation in the Spanish patient, it is unlikely that the two Ile179Met mutations are identical by descent. In this context, it is pertinent to note that the Ile179Met substitution could also have been mediated by gene conversion, templated either by the *CSH1*, *CSH2* or *CSHP1* gene.

Four novel *GHI* missense mutations (Ala13Val, Arg19His, Phe25Tyr and Ser95Arg) were also identified in the Beninese samples. However, only Phe25Tyr could have been templated by gene conversion, the potential donor sequence being the *GH2* gene. None of these missense variants appears to have any detrimental effect on GH function, as measured by a STAT5-luciferase reporter gene assay (Table 3), or affect the secretion of the protein as compared to the wild-type (Table 3). Details of the evolutionary conservation of the residues harbouring these missense variants and their predicted interactions with the GHR can be found in the Supplementary Results section.

One of the more common *GHI* variants in the Benin population (with a frequency of 5%) involves the concomitant introduction of five nucleotide sequence changes (see Fig. 1) into the promoter/5'UTR. These changes appear to have arisen as a consequence of gene conversion templated either by the *CSH1* or *CSH2* gene. The minimum extent of the conversion event was -53 to -39 whilst the maximum was -112 to +9 (*CSH1*) or -123 to +9 (*CSH2*), depending upon the identity of the donor sequence (Fig. 1). Three of the five nucleotide changes were located within the vitamin D receptor binding site (Fig. 1), a negative regulatory element in the human *GHI* gene (Seoane et al., 2002). In terms of the component nucleotide sequence changes, it is therefore identical to the gene conversion event previously reported in a healthy European of above average height (individual S121; Millar et al., 2003).

Whereas the solitary example of the European gene conversion occurred on promoter haplotype H22, its Beninese counterpart occurred at polymorphic frequency and invariably on promoter haplotype H43. Since H22 differs from H43 at four distinct positions within the promoter region viz. -308, -301, -6 and -1 (these bases being T, T, A and A respectively in H22 and G, G, G and T in H43), it follows that the two haplotypes cannot be readily inter-converted by sequential single base-pair substitutions. In addition, whilst the gene conversion in the Benin population was

found in *cis* with A+10G in all cases and with C+438T (Ala13Val) in 17/18 cases, the European gene conversion was associated with G-373A. Both gene conversions were found to be in *cis* to a T at +1169, the frequency of which was 0.37 (115/312) in Europeans as compared to 0.03 (10/326) in the Beninese population. We therefore surmise that the gene conversion event in question must have occurred independently on at least two separate occasions in the European and Beninese populations.

In order to determine whether the linked A+10G substitution contributes to the high expression of the gene conversion allele, the two variants were studied both individually and in combination. Luciferase reporter gene assays were performed in sextuplicate on three separate occasions; data were normalised as described above and the haplotype-associated activities of pGL3H43GC, pGL3H43+10G and pGL3H43GC+10G were divided by those of pGL3H43. This ensured that (i) the mean activity of H43 equalled unity in each comparison and (ii) the derived activity values for H43GC, H43+10G and H43GC+10G could be given as -fold changes relative to their respective background promoter haplotypes (Table 4). Irrespective of the background promoter haplotype, it would appear that the gene conversion event gives rise to an increase in promoter activity. By contrast, the A+10G substitution serves to reduce promoter activity when associated with haplotype H43, thereby partially counteracting the effect of the gene conversion.

Analysis of the exon 3 *GHR* deletion polymorphism was performed on 156 British and 154 Beninese DNA samples as described by Horan et al. (2006) (Figure 2). The frequency of the *GHR* d3 allele was found to be significantly higher in the Benin population than in the British sample (47% vs. 28%,  $\chi^2=22.64$ , 1 d.f.,  $p<0.0001$ ) and consequently 70% of the Beninese population possess either a d3/d3 or fl/d3 genotype as compared to 47% of Britons. The observed *GHR* d3 genotype frequencies in the British population were in broad agreement with those previously reported from other European populations (Dos Santos et al., 2004; Binder et al., 2006; Pilotta et al., 2006; Carrascosa et al., 2006; Blum et al., 2006). Genotype frequencies did not deviate significantly from Hardy-Weinberg equilibrium in either the British or Beninese groups. No significant association was apparent between *GHR* genotype and *GHI* promoter haplotype or any of the missense variants detected in this study.

#### 4. Discussion

Growth hormone (GH), encoded by the *GHI* gene, plays an important role in human development, growth and metabolism (Kaplan 1999). The biological effects of GH are mediated by binding to a GHR dimer present on the cell surface, thereby initiating a signalling cascade which results in an increased plasma level of IGF-1. We and others have previously documented an unparalleled level of *GHI* gene variation in people within the range of normal stature (Giordano et al., 1997; Wagner et al., 1997; Hasegawa et al., 2000; Horan et al., 2003; Millar et al., 2003; Giordano et al., 2006; Giordano et al., 2008). We have also expressed the view that selection for variation in stature alone is insufficient to explain this degree of genetic variability and that selection for multiple GH-related phenotypic traits has probably been responsible for creating it (Millar et al., 2003). In an attempt to relate the extensive genetic variation found in the *GHI* gene to GH structure, function and expression, a group of individuals from Benin in West Africa was screened both for mutations and polymorphisms in their *GHI* genes.

The results of this analysis have indicated that the human *GHI* gene is at least as polymorphic in the West-African population as in the European population. This is exemplified by the haplotype diversity observed for the 15 SNPs located in the *GHI*

promoter, which was 88.2% in the Beninese population as compared to 87.6% in the Europeans. This is consistent with the recently published findings of Sedman et al. (2008) who also studied *GHI* gene diversity in individuals (Mandenka) from West Africa.

Four novel coding sequence variants (Ala13Val, Arg19His, Phe25Tyr and Ser95Arg) were identified here, three of which (Ala13Val, Phe25Tyr and Ser95Arg) were found to occur at a frequency of >1%. Functional analysis of these variants revealed that they were indistinguishable from wild-type GH in terms of (i) their ability to activate the JAK-STAT signalling pathway and (ii) their secretion from GC cells.

The most frequent promoter haplotype in the Beninese population was found to be H1 (26%) which is also known to be the predominant haplotype in Britons (33%; Horan et al., 2003). This finding is consistent with an ancient origin for H1 that must have preceded the divergence of the two ethnic groups over the last ~150,000 years (Wolf et al., 2008). Of the 37 promoter haplotypes found in the Beninese population, 23 were found to be unique (Table 2) to this population whereas 14 (including H1) have also been noted in Britons.

Assessment of the correlation between haplotype frequency and expression level previously revealed that those promoter haplotypes associated with a reduced level of reporter gene expression tend to be more prevalent in the British population (Horan et al., 2003), possibly as a consequence of selection. This was not however found to be the case in the Beninese population. Indeed, the average expression level of the *GHI* promoter haplotypes was significantly higher in the Beninese than in the European population sample. This discrepancy may be explicable in terms of differential selection operating in the two populations with respect to the phenotypic consequences of reduced *GHI* gene expression. In view of the relatively low proportion of variance in adult body height explicable by *GHI* gene expression variability (Horan et al. 2003), differential selection appears very unlikely to have been mediated by stature alone but could instead reflect other factors e.g. fetal development, nutritional balance between mother and child, resistance to starvation, infection or trauma (Millar et al., 2003).

The gene conversion polymorphism detected in the present study constituted ~5.5% of Beninese *GHI* alleles. Although polymorphic gene conversions in human genes are generally rare, they are not wholly unprecedented (e.g. Ohta 1999; Matsubara et al., 2001; Ogasawara et al., 2001). Indirect evidence has been previously presented for their probable occurrence in the human *GHI* gene (Giordano et al., 1997). The gene conversion reported here resulted in the partial replacement of the wild-type *GHI* promoter sequence, specifically introducing five nucleotide substitutions [including three within the negative vitamin D response element (VDRE); Seoane et al., 2002]. The European version of this mutation, present on haplotype H22, was previously found to drive reporter gene expression to  $182\pm 6\%$  that of the wild-type H22 construct (Millar et al., 2003). Since we have shown that reporter gene expression is also elevated to  $174\pm 4\%$  in the context of haplotype H43 (Table 4), it appears likely that the gene conversion-derived alleles increase the expression of the *GHI* gene in a haplotype-independent fashion, perhaps by perturbing the putative negative VDRE regulatory element. In the West-African group, the gene conversion was associated exclusively with haplotype H43, consistent with the view that all extant copies of the allele are identical by descent in this population. H43 is the second highest of the novel African promoter haplotypes in terms of its *in vitro* expression level – it is however unclear whether this is simply coincidental or whether this high level of

expression has perhaps contributed to the spread of the haplotype through the population. This question notwithstanding, it is clear that the gene conversion event has recurred independently in European and African populations.

The gene conversion was found to occur in *cis* to a 5' UTR substitution (A+10G) in all cases and the Ala13Val (C+438T) substitution in all but one case. Neither of these nucleotide substitutions could have been mediated by gene conversion since the various paralogous genes at the GH locus all contain an adenine (A+10G) or cytosine (C+438T) at the analogous locations. This suggests that the three lesions did not occur contemporaneously as part of the same mutational event but rather that A+10G and C+438T probably occurred on a chromosome already bearing the gene conversion. This assertion is, however, based on the sequence alignment of the paralogous GH genes from a European, not a West-African, population; the nucleotides at these positions may well be different in Africans and Europeans (although it should be pointed out that this was not found by Sedman et al., 2008). Although the Ala13Val variant has no effect on GH function or secretion, the H43 haplotype bearing the +10G variant is associated with a reduction in *GHI* promoter activity to ~80% of that of the +10A-bearing H43 haplotype. The presence of the +10G variant in *cis* to the gene conversion serves to reduce promoter activity from 1.74- to 1.33-fold of the +10A-bearing H43. Since H43 has the second highest *in vitro* expression level of all haplotypes (1.35-fold higher than H1), the presence of the +10G allele simply reduces the level of promoter activity associated with H43 to that seen with H1.

Analysis of the *GHR* exon 3 deletional (d3) polymorphism in the Beninese population indicated that the frequency of the d3 allele was rather higher than in the British population (48% vs. 28%). The d3 allele has been shown to be associated with increased GH responsiveness (Dos Santos et al., 2004) and was found to be positively correlated with postnatal growth velocity and near-final height in a group of 115 healthy adolescents (Jensen et al., 2007). Atzmon et al. (2006) found that heterozygotes for the *GHR* d3 allele exhibited reduced insulin sensitivity as compared to *GHR* fl homozygotes, suggesting that the *GHR* d3 allele may also play a role in metabolism as well as in growth velocity.

Our results suggest that both high expressing *GHI* promoter haplotypes and the *GHR* d3 allele could have been selected for in the West-African population, possibly in response to persistent under-nutrition (Luke et al., 2001). Starvation is known to result in an increase in circulating GH level secondary to an increase in GH pulse frequency and amplitude (Hartman et al., 1992). Coupled with reduced insulin secretion, this leads to increased lipolysis and ketogenesis, insulin resistance and conservation of glucose and protein (Nørrelund 2005). The maintenance of a high level of circulating GH in malnourished individuals may therefore have been of selective value. However, increased lipolysis gives rise to elevated levels of circulating free fatty acids which have been shown, at least *in vitro*, to decrease expression of GHR by ~50% (Thimmarayappa et al., 2006). Decreased amounts of GHR on the cell surface would be expected to lead to reduced secretion of IGF1. The presence of at least one *GHR* d3 allele in 70% of the West African population may serve to ameliorate the effects of decreased expression of the GHR by virtue of its increased responsiveness to GH (~30% greater than for *GHR* fl homozygotes; Dos Santos et al., 2004) and IGF1 levels may consequently be near normal.

The data from our *in vitro* *GHI* promoter expression study suggest that the West-African population may well be characterized by an increased level of circulating GH. This is compatible with the *in vivo* data of Wright et al. (1995) who found that the circulating GH level was significantly higher in African-Americans than in their

European-American counterparts. Were the frequency of the *GHR* d3 allele in African-Americans to be comparable to that seen in the West-African population, then those African-Americans possessing the *GHR* d3 allele may be more responsive to GH, leading to increased circulating levels of IGF1. However, in studies of adults and post-pubertal adolescents, no significant differences in IGF1 levels were noted between African-Americans and European-Americans (Wright et al., 1995; Arslanian and Suprasongsin, 1996; Arslanian 1998) although IGF1 levels were found to be significantly higher in the African-American group in pre-pubertal children (Arslanian 1998). All such comparisons between African-Americans and European-Americans should however be viewed with caution both because of genetic admixture (Parra et al., 1998) and because of the lifestyle differences between the two populations. Further study of GH and IGF1 levels is therefore necessary in the indigenous West-African population in order to account for the initial intriguing findings in African-Americans.

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**Figure legends**

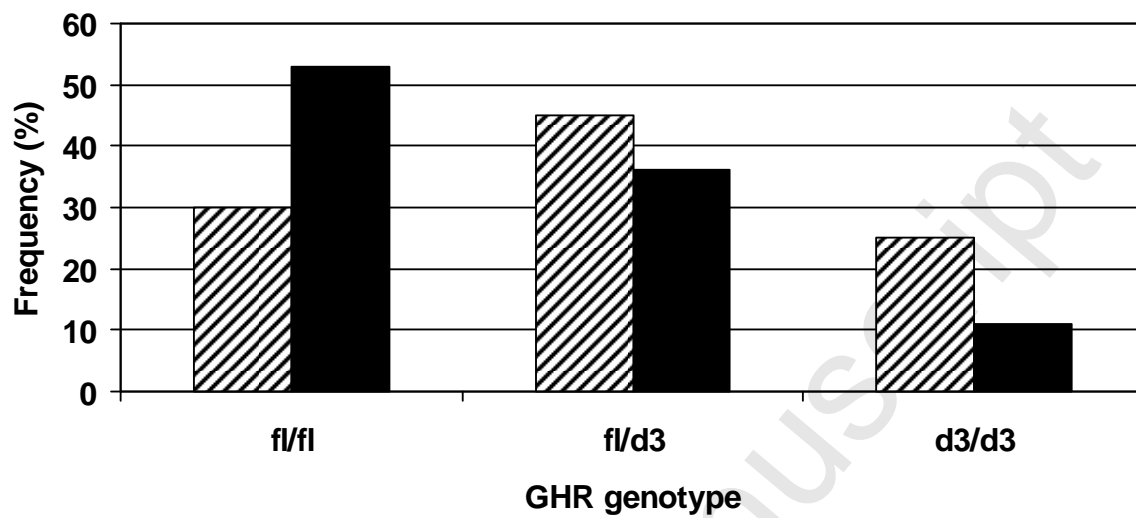
**Fig. 1** A *GHI* gene conversion identified in the Beninese population. The promoter sequence differs from the wild-type *GHI* gene sequence at five nucleotide positions between -39 and -53 where it corresponds precisely to the sequence of the paralogous *CSH1* and *CSH2* genes. The vitamin D response element (Alonso et al., 1998; Seoane et al., 2002) in the human *GHI* gene is marked in bold.

**Fig.2.** *GHR* exon 3 deletion (d3) polymorphism genotype frequencies in the Beninese (hatched bars) and British (solid bars) populations.

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Figure 2.



**Table 1.** Location and frequency of *GHI* variants occurring in the Beninese population sample at a frequency of >1%

Variant <sup>a,b</sup>	Entrez SNP	Location	Allele frequency (%)
delCT -525/-526	-	Promoter	2.2
A-360G	<b>rs61761357</b>	Promoter	13.5
T-352G	-	Promoter	3.1
G-261C	<b>rs61761358</b>	Promoter	6.4
C-227T	-	Promoter	8.9
Gene conversion <sup>c</sup>	-	Promoter/5' UTR	5.5
A+10G	-	5' UTR	6.1
A+69G (Thr3Ala <sup>d,e,f</sup> )	<b>rs2001345</b>	Exon 1	11.0
G+140A	-	Intron 1	1.2
G+144A	-	Intron 1	1.2
G+192C	<b>rs2001344</b>	Intron 1	12.9
T+281C <sup>e</sup>	<b>rs3744287</b>	Intron 1	3.4
C+438T (Ala13Val)	-	Exon 2	5.2
C+472A (Ala24Ala)	-	Exon 2	1.2
T+474A (Phe25Tyr)	-	Exon 2	1.2
C+596T	<b>rs57431470</b>	Intron 2	1.8
T+986A (Ser95Arg)	-	Exon 4	2.2
G+1070A (Thr123Thr) <sup>e</sup>	<b>rs41295043</b>	Exon 4	2.8
G+1098T	<b>rs61735351</b>	Intron 4	5.2
G+1136A	<b>rs61735352</b>	Intron 4	4.9
T+1169A <sup>e,g</sup>	<b>rs2665802</b>	Intron 4	3.1
G+1215A	<b>rs61735355</b>	Intron 4	1.8
T+1231A	<b>rs61735356</b>	Intron 4	1.5
delA+1246	-	Intron 4	1.2

<sup>a</sup> Nomenclature recommended by den Dunnen and Antonarakis (2000)

<sup>b</sup> Numbering relative to the transcription initiation site at +1 (GenBank accession number J03071)

<sup>c</sup> Gene conversion to *CSH1* (minimum -53 to -39; maximum -112 to +9) or *CSH2* sequence (minimum -53 to -39; maximum -123 to +9)

<sup>d</sup> Previously termed Thr-24Ala by ourselves and others

<sup>e</sup> Previously identified in the European population (Esteban et al., 2007)

<sup>f</sup> Previously identified in the Japanese population (Miyata et al., 1997)

<sup>g</sup> Previously identified in the Japanese population (Hasegawa et al., 2000)

**Table 2.** *GHI* promoter haplotypes (as defined by Horan et al., 2003) found in the Beninese population: *in vitro* expression assay data and population frequencies

Haplotype acronym	Haplotype <sup>a</sup>	Luciferase activity $\pm$ SEM	Benin n = 326 (%)	European <sup>b</sup> n = 606 (%)
H1	GGGGGGTATGAAGAAT	100.0 $\pm$ 2.3	85 (26.0)	191 (31.5)
H8	GGGTTGTAGGGAGAAT	103.0 $\pm$ 9.8	51 (15.6)	17 (2.8)
H23	GGGGGGTAGGAAGAAT	33.0 $\pm$ 3.2	32 (9.8)	5 (0.8)
H42	GGGGGGTAGGACCAAG	98.7 $\pm$ 11.1	23 (7.0)	-
H43	GGGGGGTATGGTGAAT	135.3 $\pm$ 17.8	18 (5.5)	-
H44	GGGTTGTAGGACCAAT	135.2 $\pm$ 12.5	15 (4.6)	-
H35	GGGGGGTAGGACCAAT	83.6 $\pm$ 8.0	13 (3.9)	2 (0.3)
H45	GGGGGGTAGGGAGGAT	138.8 $\pm$ 11.5	11 (3.3)	-
H2	GGGGGTTAGGGAGAAT	97.2 $\pm$ 9.5	9 (2.7)	91 (15.0)
H7	GGGGGTTAGGGTGAAT	61.1 $\pm$ 3.6	9 (2.7)	13 (2.1)
H46	GT-GGGTATGGAGAAT	136.7 $\pm$ 13.1	7 (2.1)	-
H47	GT-GGGTAGGGAGAAT	119.9 $\pm$ 12.2	5 (1.5)	-
H48	GGGTTGTGGGACCAAT	77.6 $\pm$ 6.5	5 (1.5)	2 (0.3)
H49	GG-GGTTAGGGTGAAT	111.1 $\pm$ 9.0	5 (1.5)	-
H50	GGGGGGTAG-ACCAAG	129.9 $\pm$ 12.1	4 (1.2)	-
H51	GGGGGGTAGGGAGAAT	133.0 $\pm$ 14.4	4 (1.2)	-
H3	GGGTTGTAGGAAGAAT	37.1 $\pm$ 3.3	3 (0.9)	41 (6.8)
H17	GG-GGTTAGGGAGAAT	54.6 $\pm$ 3.1	3 (0.9)	6 (1.0)
H52	GGGGGGTAG-ACCAAT	121.9 $\pm$ 13.8	3 (0.9)	-
H20	GGGGGGTAG-AAGAAT	75.6 $\pm$ 5.0	2 (0.6)	3 (0.5)
H53	GGGGGGTAGGGTGAAT	119.8 $\pm$ 14.5	2 (0.6)	-
H54	GGGTTGTGGGAGAAT	85.1 $\pm$ 13.2	2 (0.6)	-
H15	GGGTTGTAGGGTGAAT	59.6 $\pm$ 5.2	1 (0.3)	12 (2.0)
H22	GGGTTGTATGAAGAAT	100.4 $\pm$ 8.1	1 (0.3)	3 (0.5)
H55	GGGGGGTATGATGAAT	135.3 $\pm$ 4.9	1 (0.3)	-
H56	GGGGGGTGGGAGAAT	81.5 $\pm$ 4.5	1 (0.3)	-
H57	GGGGGTTAGGACCAAT	134.0 $\pm$ 10.6	1 (0.3)	1 (0.2)
H58	GGGGGTTAGGGCCAAT	152.8 $\pm$ 13.1	1 (0.3)	-
H59	GGGGGGTGTGAAGAAT	169.8 $\pm$ 13.8	1 (0.3)	-
H60	GT-GGGTAG-GAGAAT	136.5 $\pm$ 6.2	1 (0.3)	-
H61	GGGTTGTAGGACCAAG	111.0 $\pm$ 9.1	1 (0.3)	-
H62	GGGGGGTAGGACCGCG	130.3 $\pm$ 5.3	1 (0.3)	-
H63	GGGGGGTAGGGCCAAG	124.0 $\pm$ 4.6	1 (0.3)	-
H64	GGGGGGTAGGGAGGAG	122.5 $\pm$ 6.9	1 (0.3)	-
H65	GT-TTGTAGGGAGAAT	140.1 $\pm$ 10.7	1 (0.3)	-
H66	GGGGGGTGGGACCAAT	94.0 $\pm$ 5.3	1 (0.3)	-
H67	GGGGGGTAG-GTGAAT	118.0 $\pm$ 7.7	1 (0.3)	1 (0.2)

<sup>a</sup> order of SNPs as described in Table 1 of Horan et al., (2003) and Supplementary Table 2.

<sup>b</sup> Haplotypes identified in Europeans (Horan et al., 2003; Lewis et al. 2004; unpublished results). NB. Only 388 of the 606 haplotypes (64.0%) are listed; the remaining European haplotypes were not found in the Beninese population sample.

**Table 3.** Analysis of functional activity and secretion *in vitro* of GH coding sequence variants

Variant	GH function (EC <sub>50</sub> )				GH secretion			
	EC <sub>50</sub> nM	SEM	n	p <sup>a</sup>	Mean %	SEM	n	p <sup>a</sup>
Wild-type <sup>b</sup>	2.98	0.41	6	-	100	13	4	-
Ala13Val	2.43	0.09	3	ns	87	5	4	ns
Arg19His	3.77	0.64	3	ns	74	5	4	ns
Phe25Tyr	2.85	0.45	3	ns	77	3	4	ns
Ser95Arg	3.90	0.22	3	ns	125	5	4	ns

<sup>a</sup>p values from a comparison with wild-type using ANOVA.

<sup>b</sup>wild-type refers to GH variant 1 protein (accession number NP\_000506).

ns: not significant. SEM: Standard error of the mean.

Mean %: mean calculated as a percentage of the average wild-type value.

EC<sub>50</sub> nM is the median effective concentration (required to induce a 50% effect).

**Table 4.** Luciferase reporter gene assay-based expression activity of different combinations of haplotype 43 with the gene conversion and A+10G sequence variants

	Mean	SEM	n	p <sup>a</sup>
H43	1.000	0.056	18	-
H43 and gene conversion (H43GC)	1.744	0.100	18	<0.0001
H43 and A+10G (H43+10G)	0.805	0.058	18	0.022
H43 and gene conversion and A+10G (H43GC+10G)	1.325	0.047	18	<0.0001

<sup>a</sup> p values from a comparison with haplotype H43 using a Student t-test.