Absence of the genetic variant Val\(^{79}\)Met in human chorionic gonadotropin-beta gene 5 in five European populations

Min Jiang\(^{1,8}\), Marja-Liisa Savontaus\(^2\), Henrik Simonsen\(^{3,7}\), Catherine Williamson\(^4\), Roman Müllebach\(^4\), Jörg Gromoll\(^5\), Nicole Terwort\(^5\), Maria Alevizaki\(^6\) and Ilpo Huhtaniemi\(^{1,4}\)

Departments of \(^1\)Physiology and \(^2\)Medical Genetics, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland, \(^3\)Department of Clinical Biochemistry, Statens Serum Institute, DK-2300 Copenhagen, Denmark, \(^4\)Institute of Reproductive and Developmental Biology, Faculty of Medicine, Imperial College, Hammersmith Campus, Du Cane Road, London W12 0NN, UK, \(^5\)Institute of Reproductive Medicine, University of Münster, D-48129 Münster, Germany and \(^6\)Department of Medical Therapeutics, Athens University School of Medicine, Athens, Greece

\(^7\)Present address: Department of Neonatology, Rigshospitalet, DK-2100 Copenhagen, Denmark

\(^8\)To whom correspondence should be addressed at: Department of Physiology, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland. E-mail: min.jiang@utu.fi

Chorionic gonadotropin (CG) is an essential signal in establishment and maintenance of pregnancy in humans and higher primates. A G-to-A transition in exon 3 of human CG\(^b\) gene 5, changing the naturally occurring valine residue to methionine in codon 79 (Val\(^{79}\)Met) has been reported at carrier frequency 4.2% in a random population from the Midwest of the United States. The biological activity of the variant hCG was similar to that of wild-type (WT) hCG. However, the Val\(^{79}\)Met \(\beta\)-subunit displayed impaired ability to assemble with \(\alpha\)-subunit, and the amount of hCG \(\alpha/\beta\) heterodimers formed and secreted by transfected cells was seriously impaired in the previous study. Because of these functional implications we found it important to study the occurrence of the Val\(^{79}\)Met hCG\(^b\) variant in other populations. By using a PCR–RFLP method, a search for the Val\(^{79}\)Met hCG\(^b\) variant was carried out on a total of 580 DNA samples from five European populations (Finland, Denmark, Greece, Germany and the UK). The results demonstrated an absence of the polymorphism in these populations. Hence, the naturally occurring variant (Val\(^{79}\)Met) of the hCG\(^b\) gene 5, found previously at high frequency in the US, is clearly less common, or absent, in the European populations studied.

**Key words:** genetic variant/hCG\(^b\)5/PCR

---

**Introduction**

Of the four glycoprotein hormones, chorionic gonadotropin (CG), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), CG is expressed in the placenta, and the other three in the anterior pituitary gland. Except for humans, higher primates and equines, this placentally expressed hormone has not been found in other mammalian orders (Maston and Ruvolo, 2002). There are 7 \(CG\(^b\)\) genes but only a single \(LH\(^b\)\) gene in the human genome, and they are located in tandem repeats on chromosome 19 (Boorstein et al., 1982; Talmadge et al., 1983; 1984). Of the homologous hCG\(^b\) genes, number 5 is usually most highly expressed during the first trimester of pregnancy (Miller-Lindholm et al., 1997). hCG is maximally secreted from placental trophoblast cells during the first trimester to stimulate the corpus luteum of pregnancy to produce progesterone and maintain pregnancy.

Single base-pair mutations in the \(\beta\)-subunit genes of LH, TSH and FSH can cause clinical disorders. Dysfunction of all glycoprotein hormones would be expected following mutations of the common \(\alpha\)-subunit. Such a genetic defect in the human could be lethal, which may explain why no \(\alpha\)-subunit mutations, even in heterozygous form, have so far been detected. The only alteration so far reported in the \(\alpha\)-subunit protein is a single Glu\(^{59}\)Ala substitution, apparently due to somatic mutation in a malignant tumor, giving rise to an ectopically secreted hCG \(\alpha\)-subunit with anomalously high molecular weight that failed to associate with the \(\beta\)-subunit (Nishimura et al., 1986). Somewhat surprisingly, mice with targeted disruption of the common \(\alpha\)-subunit gene are viable, though hypothyroid and hypogonadal (Kendall et al., 1995), but this species does not have a \(CG\(^b\)\) gene in its genome.

The only human loss-of-function mutations of the \(LH\(^b\)\) subunit (Glu\(^{59}\)Arg, Gly\(^{36}\)Asp) have been reported in two males with delayed puberty, low testosterone and arrested spermatogenesis (Weiss et al., 1992; Valdes-Socin et al., 2003). The homozygous LH\(^b\) mutation (Gln\(^{54}\)Arg) was found to encode a hormone that was immunologically active but totally devoid of bioactivity. The other newly discovered human LH\(^b\) mutation (Gly\(^{36}\)Asp) presented with undetectable LH immunoreactivity and elevated FSH. Two mutations (Trp\(^{8}\)Arg and Ile\(^{15}\)Thr) were identified in the N-terminal region in a healthy woman with an immunologically anomalous form of LH (Pettersson et al., 1992). This variant LH (V-LH) molecule subsequently appeared to be a common polymorphism with highly variable carrier frequency (0–43.5%) in different populations (Themmen and Huhtaniemi, 2000). In comparison with WT-LH, it has higher bioactivity in vitro but shorter half-life in circulation (Themmen and Huhtaniemi, 2000). We later found an additional
eight mutations in the V-LHβ promoter sequence that could contribute to the altered physiological activity of the LH variant molecule (Jiang et al., 1999). Additional LHβ mutations include one in the signal peptide (Ala13Thr), with functional consequences for signal transduction in LH-responsive cells in vitro (Jiang et al., 2002), and a Gly102Ser mutation (Liao et al., 1998, 2002). Functional studies on this mutation are controversial. Conspicuously, the latter two mutations (Ala13Thr and Gly102Ser) seem to be restricted to certain populations only. The Ala13Thr mutation in the signal peptide of LHβ was only detected from three heterozygous individuals out of 100 DNA samples from Rwanda (Jiang et al., 2002). The Gly102Ser mutation has been found from infertile and subfertile patients in the Singapore Chinese population (Liao et al., 1998, 2002; Lamminen et al., 2002).

Several homozygous or compound heterozygous hFSHβ mutations (Val4Stop, Tyr4Stop and Cys64Arg) have been described in three males and four females with isolated FSH deficiency (Thiemmen and Huhtaniemi, 2000). In addition, one female with primary amenorrhea and infertility has been reported due to 2 bp deletion in codon 61 of FSHβ, producing a frame-shift mutation (Matthews et al., 1993). A total of five mutations of the TSHβ subunit gene have been described worldwide as cause of low TSH with congenital hypothyroidism (Sertedaki et al., 2002).

Conspicuously, there are no reports on mutations of hCGβ. Only one genetic variant identified in hCGβ gene 5 (a G-to-A transition in exon 3), changing valine to methionine in codon 79 (Val79Met) has been reported (Miller-Lindholm et al., 1999). Heterozygotes for this mutation were found at a remarkably high carrier frequency of 4.2% in a random population analyzed from the Midwest of the United States. Although the variant αβ dimer did not display alteration of biological activity, the dimerization of α and β subunits was hampered, implying the possibility of functional consequences of the mutation. We found it therefore important to assess the frequency of the Val79Met hCGβ mutation in other populations.

Materials and methods

Subjects
A total of 580 genomic DNA samples were collected anonymously and randomly from apparently healthy donors screened for metabolic and endocrinological diseases, or collected for anthropological studies, in populations from Finland (n = 125), Denmark (n = 152), Greece (n = 100), Germany (n = 100) and UK (n = 103). Appropriate permissions for all sample collections and analyses for this study were obtained from local ethical committees.

Amplification of the hCGβ5 gene by polymerase chain reaction (PCR)
DNA amplification was carried out using PCR with specific primers designed on the basis of known sequence of the hCGβ gene 5, and selected with specific mismatches in order to discriminate between hCGβ gene 5 and the other highly homologous hCGβ and LHβ genes (Fiddes and Talmadge, 1984; Miller-Lindholm et al., 1999). First, a 444 bp PCR fragment was amplified using forward and reverse primers (hCG5ex3F and hCG5ex3R) (Table I and Figure 1), each at 0.4 µmol/L, in a total reaction volume of 50 µL containing thermostable DNA polymerase (1 unit), deoxynucleotide triphosphates (dNTPs, 0.4 mmol/L of each), in buffer containing KCl (50 mmol/L), Tris—HCl (10 mmol/L, pH 8.8), Triton X-100 (0.1%) and MgCl2 (1.5 mmol/L). Thirty-five PCR cycles were performed as follows: denaturation (96°C, 1 min), annealing (57°C, 1.5 min), extension (72°C, 1.5 min). DNA polymerase (Dynazyme™ I (Finzymes OY, Espoo, Finland) was added after the initial denaturation step (5 min). The PCR products were purified using electrophoresis on agarose gel prior to the GFX™ PCR DNA and gel band purification kit (Amersham) and submitted subsequently for sequencing (see below).

Table I. Nucleotide sequences of primers used in PCR, site-directed mutagenesis

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Oligonucleotide sequence 5′ → 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCG5ex3F</td>
<td>TCTGAGACCTGTGGGGGCACA</td>
</tr>
<tr>
<td>hCG5ex3R</td>
<td>GAGCCCAACAAAGACTCTCT</td>
</tr>
<tr>
<td>V79M sense</td>
<td>GGGTGAAACCCCATGTTCTCTTACG</td>
</tr>
<tr>
<td>V79M antisense</td>
<td>CGTAGGAGCACCATGGGTCACGCC</td>
</tr>
</tbody>
</table>

Figure 1. (A) Schematic presentation of the hCGβ5 gene and positions of the oligonucleotide primers used in PCR. The filled boxes correspond to sequences encoding the three exons. The arrow indicates the GTG→ATG mutation at position 1432 in exon 3 and the NcoI cleavage site. The mutation changes the valine residue to methionine at codon 79. hCG5ex3F, hCG5ex3R depict the approximate positions of the PCR primers (see Table I). (B) Agarose gel electrophoresis of the NcoI restriction digestion products of the hCGβ subunit amplified by PCR using primers hCGex3F and hCGex3R. MSM, molecular size markers; lane 1, undigested PCR product (444 bp); lane 2, WT hCGβ5 sample (444 bp, no NcoI cleavage site); lane 3, homozygous mutant G-to-A (Val79Met) in exon 3 of hCGβ gene 5 (248 and 196 bp fragments cleaved by NcoI).

Site-directed mutagenesis for preparation of a positive control DNA
Because the positive genomic (Val79Met) DNA was not available, we generated a positive control mutant DNA for this study. First, the 444 bp PCR fragment of the WT hCGβ gene 5 was subcloned into the pCR®4BluntTOPO™ vector according to the manufacturer’s instructions (Invitrogen Corporation, Carlsbad, CA). Then the pCR®4BluntTOPO™ vector containing the 444 bp CBG fragment was used for site-directed mutagenesis to generate the G-to-A transition in the insert. The mutant hCGβ gene 5 was constructed by site-directed mutagenesis (Quickchange™ Site-Directed Mutagenesis Kit, Stratagene Cloning System, La Jolla, CA). A pair of primers
was designed to incorporate the G-to-A transition to produce a methionine residue in place of the WT valine (Table I) (Miller-Lindholm et al., 1999). The PCR reactions were carried out in a total volume of 50 µl containing 2.5 U Pfu Turbo® DNA polymerase (Stratagene, La Jolla, CA) in 1X native Pfu Turbo® PCR buffer solution consisting of 20 mM Tris–HCl (pH 8.75), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100, 100 µg/ml BSA, 10 mM KCl, 4 mM each of dATP, dCTP, dGTP and dTTP, and 25 ng of WT hCG plasmid. PCR amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA) using a program of 95°C for 1 min, followed by 15 cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 10 min. XL1-Blue Super competent cells (Life Technologies) were transformed with the pCR®4Blunt-TOPO® vector containing the mutated hCGβ gene 5 insert. For the mutagenesis and transformation controls, cells were spread on LB–ampicillin agar plates containing 80 µg/ml X-gal and 20 mM IPTG. The positive clones were grown overnight in 2 ml LB medium, purified by the alkaline lysis method and checked for presence of the G-to-A change in the 444 bp insert by restriction enzyme digestion and sequencing. Oligonucleotides used for PCR and site-directed mutagenesis were prepared by TAG Copenhagen A/S (Copenhagen, Denmark).

Testing of the positive control DNA and screening for the Val⁷⁹Met variant of the hCGβ 5-subunit by PCR and restriction fragment length polymorphism (RFLP)

The mutated plasmids containing the G-to-A transition in the hCGβ gene 5 were used as template in PCR to amplify the variant hCGβ gene 5 fragments. The primers used, and the reaction conditions of PCR, were the same as when amplifying the 444 bp PCR products from genomic DNA samples. If the 444 bp length PCR fragment comprised a G-to-A transition at position 1432 in exon 3 of the hCGβ gene (GenBank accession no. X00265; Talmadge et al., 1984), this nucleotide change created a cleavage site for restriction enzyme NcoI (Promega). PCR–RFLP analysis of wild-type alleles yields a 444 bp fragment. In the presence of the mutation at codon 79, the NcoI recognition site is present so that in heterozygotes the bands can be identified, i.e. 444 bp (wt), 248 bp and 196 bp, whereas in homozygous mutants there are two bands of 248 bp and 196 bp (Figure 1).

The genomic DNA samples collected from Finland, Denmark, Greece, Germany and the UK were analysed. The PCR products were amplified by PCR with primers hCG5ex3F and hCG5ex3R (Figure 1) and subsequently digested by restriction enzyme NcoI to screen for the Val⁷⁹Met (a G-to-A change in exon 3) variant of hCGβ 5 subunit (Miller-Lindholm et al., 1999). Five units of NcoI were used to digest a 5 µl aliquot of the of PCR product (37°C, overnight incubation), followed by electrophoresis through a 2% agarose gel and visualization with ethidium bromide.

DNA sequencing

PCR products from several individual PCR runs, as well as the G-to-A change constructed by site-directed mutagenesis, were sequenced to confirm the genotypes, and to check for possible polymerase errors. The PCR primers hCG5ex3F, hCG5ex3R (Figure 1; Table I) and M13 forward and reverse primers were used as sequencing primers with the dideoxy chain termination method. Sequencing was performed on an ABI PRISM™ 377 DNA Sequencer (Perkin Elmer, Norwalk, CT).

Results

Validation of the detection method

The 444 bp long PCR fragment of WT-hCGβ gene 5 was subcloned into the pCR®4Blunt-TOPO® vector, then the plasmid containing the WT 444 bp fragment of hCGβ gene 5 was used in site-directed mutagenesis to generate the G-to-A transition in this fragment. The variant hCGβ gene 5 fragments were amplified from mutated plasmids by PCR. The PCR products and the plasmids from sub-cloning and site-directed mutagenesis were all subsequently used in sequencing, to confirm the genotypes. The positive results of NcoI digestion from PCR products of variant hCGβ gene 5 (Figure 1) were also confirmed by sequencing. Thus, PCR together with RFLP (NcoI) gives reliable results in this population screening study.

Absence of the Val⁷⁹Met variant in five European populations

A total of 125 Finnish, 152 Danish, 100 Greek, 100 German and 103 Irish and British DNA samples were screened, by the PCR and RFLP (NcoI) method described above, including analysis of homoygous positive controls. Except for the positive control samples, the Val⁷⁹Met variant was not found in any of the samples studied from these five populations. The difference in frequency of the mutation was statistically significant between the US and European populations (P < 0.001; Chi-square test).

Discussion

The glycoprotein hormones are composed of a common α-subunit and a unique β-subunit that determines their biological specificity. The non-covalent association of the αβ-subunits is an obligatory step for the formation of biologically active hormones. The correct folding and assembly of the heterodimer is important for the efficient secretion of biologically active hormone (Peters et al., 1984; Corless et al., 1987; Huh et al., 1994; Narayan et al., 1995). At the beginning of pregnancy, 10 to 12 days after conception, hCG production starts in the trophoblastic cells of the placenta, forming a critical signal for the maintenance of corpus luteum of pregnancy. The timely onset and subsequent sufficient production of hCG are vital for the maintenance of pregnancy. To date, unlike the number of mutations reported in the LH and FSH receptor genes, only a few sporadic cases of mutations of gonadotropin β-subunit genes have been reported (see Introduction for references, and Themmen and Huhtaniemi, 2000). It is therefore conceivable that the production of structurally and functionally altered hCG could be the cause of primary infertility or recurrent abortions. Such cases could respond to treatment with exogenous hCG.

The Val⁷⁹Met hCGβ variant studied here was first discovered at 4.2% carrier frequency (Miller-Lindholm et al., 1999). The expression of a Val⁷⁹Met β-subunit and characterization of the protein-folding phenotype of this genetic variant revealed that this β-subunit was impaired in its ability to assemble with the glycoprotein hormone α-subunit to produce αβ heterodimers. As much as 85% of the Val⁷⁹Met β-subunit synthesized was secreted as unassembled free β, while 50% or less was unassembled in cells expressing the WT β-subunit. Although the amount of hCG αβ heterodimers formed and secreted by βVal⁷⁹Met-producing cells was less than that by cells producing WT hCGβ, the hCG that was secreted as Val⁷⁹Met αβ heterodimers did not have significantly different biological activity from that of WT-hCG (Miller-Lindholm et al., 1999). When the genotypic frequency of the polymorphisms in hCGβ gene 5 was studied in 334 asymptomatic individuals and 41 infertile patients, no significant difference in frequency was found between the infertile subjects and random population (Miller-Lindholm et al., 1999). However, a subtle deficiency of bioactive hCG is possible in individuals carrying this polymorphism, and it may become clinically significant in homozygotes.

There are seven genes in the hCG gene family, three of which are expressed to some degree; while gene 5 is typically that most highly expressed during the first trimester of pregnancy, genes 3 and 8 can also be expressed in relatively high amounts. There is variation in the expression levels of each gene between individual placentas (Miller-Lindholm et al., 1997). Because the seven homologous hCGβ genes are linked as tandem repeats on chromosome
and 8. On the other hand, the Val79Met hCG genotype is viable in utero and fertile and/or capable of sustaining a pregnancy, this could be due to the up-regulation of hCG genes 3 and 8. On the other hand, the Val79Met hCG mutation hampers dimerization with the -subunit. The homozygotes of hCG Val79Met might therefore have severe hCG deficiency and be possibly infertile, and if homozygous fetuses do not survive, the genotype could consequently not be vertically transferred. However, we could not identify a single heterozygote in a total of 580 subjects from five European populations (Finland, Denmark, Greece, Germany and the UK).

There are a number of polymorphisms in the hCG genes reported as single nucleotide polymorphisms (SNPs) database (www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi). However, all of these polymorphisms occur within intronic regions, or are silent. It is unknown whether any of these polymorphisms have phenotypic effects. In this study, it seems that the occurrence of the Val79Met variant of hCG was recently found at a carrier frequency of 4.2% in the Midwest of the US is rare or absent in Finnish, Danish, Greek, German and British populations. Our differential finding was surprising and prompts the explanation that the Val79Met genetic variant only exists, possibly due to the founder effect, in certain population isolates of recent immigrants to the US. It is therefore unlikely that this mutation plays an important role in idiopathic infertility, which could be the phenotype in case of homozygosity. Further proof would necessitate large-scale analysis of samples from infertile females.

Acknowledgements

This study was supported by grants from the Academy of Finland and the European Union.

References


Submitted on June 16, 2004; accepted on July 13, 2004