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ANTIGENIC ALTERATION OF AN ANOMALOUS HUMAN LUTEINIZING HORMONE CAUSED BY TWO CHORIONIC GONADOTROPIN-TYPE AMINO-ACID SUBSTITUTIONS

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We analyzed the nucleotide sequence of the luteinizing hormone β subunit (LHβ) in a patient with an anomalous LH. This anomalous LH showed abnormal immunogenicity, but normal bioactivity, suggesting that this variance of antigenicity was caused by amino acid substitution(s). In the anomalous LH, two single amino acid substitutions, Trp(TGG) to Arg(CGG) and Ile(ATC) to Thr(ACC), were found at the codon for the 8th and 15th residue of LHβ. These two substituted amino acid residues of the anomalous LH are identical to those of chrionic gonadotropin, but not to those of LH, although the rest of the region showed the normal sequence of human LHβ. Pedigree analysis by direct DNA sequencing revealed that the parents of the patient and the healthy sister were heterozygotes for the mutation and the patient and the healthy brother were homozygotes.

Human luteinizing hormone (LH) is a member of the glycoprotein hormone family produced in the anterior lobe of the pituitary gland that also produces follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH), and chorionic gonadotropin (CG) (1). Each of these hormones is a heterodimer composed of an α subunit and β subunit. All members of this hormone family share the common α subunit which is transcribed from its single gene. However, the β subunit is specific for each hormone, and the β genes are considered to be diverse. Especially, LH and CG are very similar both functionally and structurally, although they are expressed in different tissues at different developmental stages.

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Abbreviations: LH, luteinizing hormone; TSH, thyroid-stimulating hormone; CG, chorionic gonadotropin; IRMA, immunoradiometric assay.

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Mutations of the glycoprotein hormone family have been found in TSH, FSH and LH. These mutations are substitutions producing a termination codon in the open reading frame of the β subunit genes in TSH (2), LH (3) and FSH (4) , or were those causing amino acid substitutions which break down their normal bioactivities (5). On the other hand, two pedigrees of patients with inherited anomalous LH have been reported recently by Pettersson et al. (6) and Okuda et al. (7). LH has a central role in ovaries in promoting to synthesize steroids. However, these patients did not show any abnormalities or clinical symptoms, indicating that the LH bioactivity in these patients is normal (6,7). However, the antigenicity (some epitopes) was altered, that is, it could be detected by some of the monoclonal antibodies and all polyclonal antibodies raised against the LH heterodimer, but not by specific monoclonal antibodies. Both cases with the inherited anomalous LH, followed an autosomal dominant inheritance pattern and had no clinical symptoms. In combination with the data that the antigenicity of other glycoprotein hormones in these patients is completely normal, these clinical findings lead us to conclude that this abnormal immunogenicity might be caused by nucleotide mutation(s) occurring in the coding region of the LH β gene of the patients with the inherited anomalous LH, which generate(s) the amino acid substitution(s).

Herein, we describe the DNA analysis of the inherited anomalous LH β gene by direct PCR sequencing. Two mutations in exon 2 of the patients' LH β gene were found. They resulted in amino acid changes at the 8th codon for Trp(TGG) to Arg(CGG) and at the 15th codon for Ile(ATC) to Thr(ACC). The two altered codons were the same as those of chorionic gonadotropin but not as luteinizing hormone. This anomalous LH β gene might have been generated by an alternative evolutional diversion from a common ancestor gene of the gonadotropin family.

MATERIALS AND METHODS

Subjects

The clinical history, physical findings, and endocrinologic features of the patient and her family have been described (7). In brief, the patient presented at the age of 29 years because of repeated abortion. Her menarche had occurred spontaneusly at the age of 13, followed by regular menstruation (26-28 days interval) and development of secondary sexual characteristics thereafter. She married at 24 years of age. She had experienced two spontaneous abortions in the first trimester. The karyotype of cultured peripheral leukocytes was normal (46,XX). There were no somatic anomalies. Her external genitals and vagina were normal. Hysterosalpingography showed a normally shaped uterine cavity, and both patent tubes. Her husband's karyotype was also normal (46,XY), and his semen analysis was normozoospermic. Her brother and sister were fertile as proved by their having three and two children, respectively.

The serum LH in the patient was not detected by the sensitive immunoradiometric assay (IRMA) using two monoclonal antibodies. The two monoclonal antibodies recognize epitopes present on the intact LH dimer, but not on the free subunit. The immunologically anomalous LH (IA-LH) in the patient was bioactive, as evidenced by an in vitro bioassay

for LH. The concentrations of the other pituitary hormone, such as follicle-stimulating hormone (FSH), and thyroid stimulating hormone (TSH) were within normal limits. She became pregnant as diagnosed by the detection of chorionic gonadotropin in her urine. Her pregnancy course was uneventful and she delivered a healthy female baby. When IRMA was modified by changing one monoclonal antibody with the intact LH dimer to a α-specific detection antibody, serum LH was detected, and the level increased after GnRH administration.

Preparation of genomic DNA

Blood specimens were obtained from the patient, her mother (age 67 years), father (age 67 years), sister (age 38 years), and brother (age 42 years) and DNA was isolated from peripheral-blood leukocytes(8).

Sequencing analysis of IA-LH gene

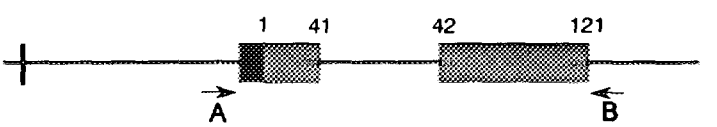
The LH β protein is encoded by a single gene that is located adjacent to several highly homologous CG β and pseudogenes on chromosome 19. Figure 1 shows a map of the LH β/CG β gene cluster and the strategy for polymerase chain reaction (PCR) amplification. The primers used contained artificial restriction enzyme sites (BamH I :primer A, EcoR I :primer B) to facilitate subcloning into the M13 vectors.

DNA amplifications

The PCR reaction was performed in 100 μl mixtures covered with 70 μl mineral oil. The reaction mixture contained 25 pmoles of each primer, 2.5 units of native Pfu DNA polymerase (Stratagene, La Jolla, CA), 500 ng of template DNA and 200 μM total dNTPs in buffer containing 20 mM Tris-HCl (pH 8.2 at 25℃), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, and 10 ng/μl BSA. Initial denaturation was done for 5 min at 94℃. Amplification was carried out for 30 cycles on a Perkin-Elmer Cetus Thermal Cycler (1 sec for transition to 94℃, 1 min at 94℃, 40 sec for transition to 68℃, 4 min at 68℃.)

Cloning of PCR product and its sequencing analysis

The PCR product was extracted once with phenol/chloroform, followed by chloroform extraction and precipitated. DNA was digested with EcoRI (20U) and BamHI (20U) at 37℃ for 3 h in 20 μl of the buffer containing 100 mM Tris-HCl (pH 7.5 at 25 ℃), 50 mM NaCl, 10 mM $MgCl_2$, and 0.025% Triton X-100. After heat inactivation (70 ℃, 15 min), the PCR products were separated by electrophoresis using a 3% NuSieve agarose (FMC Bio Products, Rockland, ME), and the amplified DNA (about 800 bp) was



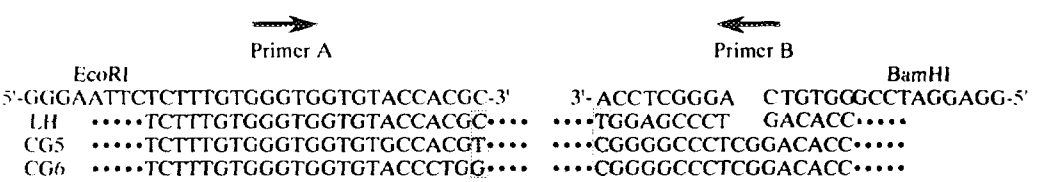


Fig.1. The primers used contained artificial restriction enzyme sites to facilitate subcloning into the M13mp19 vectors. To amplify LH β DNA, 30 cycles of denaturation (94℃, 1 min), annealing and extension (68℃, 4 min) were done on a DNA Thermal Cycler.

extracted from gel by phenol extraction (9). The DNA was ligated with EcoRI/BamHI double digested M13 mp18 or M13 mp19 vector. The ligation mixture was transfected into E.coli JM109. Recombinant phage was screened by color selection on Xgal-IPTG plate. Single-strand DNA was prepared from recombinant phage by the standard method and used as a sequencing template. DNA sequencing was performed with the chain termination method. The genotypes were confirmed by sequencing a minimum of ten clones.

Pedigree analysis of patients with IA-LH variant by PCR direct sequence

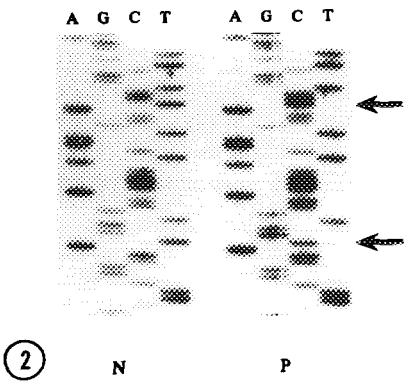
An 800-base-pair (bp) region of exon 2 and exon 3 that included the mutation site was amplified in additional family members as the same conditions as described above, sequenced by direct sequencing using the autosequencer (370A DNA Sequencer: Applied Biosystems, Inc. Foster city, CA). DNA sequencing was performed by the chain-termination method using primer A and Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Inc.).

RESULTS AND DISCUSSIONS

Analysis of nucleotide sequence

LH and CG are very similar functionally and structurally, and bind to the same testicular and ovarian receptors to promote spermatogenesis and ovulation by stimulating the testis and ovaries to synthesize steroids (10). These two genes share the same α subunit encoded in a single gene and LHβ and CGβ subunit genes specific for each hormone are structurally similar. They have evolved from an ancestral LHβ subunit to form a gene cluster on the proximal region of the short arm of human chromosome 19 in the human genome (11,12). In this gene cluster region, human LHβ is encoded by a single gene (12). On the other hand, three active chorionic gonadotropin genes and their three pseudogenes are located in the region adjacent to the LHβ gene (12,13) . The patients with inherited anomalous LH show normal bioactivity, with the exception of abnormal epitope(s) (7). Therefore, we expected the mutation to be located in the coding region of LHβ gene.

For the sequencing analysis of LHβ gene, exons 2 and 3 of the anomalous LHβ gene were amplified by PCR from leukocyte genomic DNA, and we subcloned these PCR products into plasmid vectors. Ten clones isolated from the patient's and normal DNA were sequenced. We avoided the PCR amplification of CGβ genes and its pseudogenes which is caused by the sequence similarity between LHβ and CGβ genes, as primer sequences. In the patient's LHβ gene, two single-base substitutions were identified, and sequenced (Fig.2). The first substitution from TGG(Trp) to CGG(Arg) was found at the 8th codon amino acid and the second mutation was also the single base substitution from ATC(Ile) to ACC(Thr) at the 15th codon. The changed codon CGG(Arg) and ACC(Thr) are specific for the sequence of the CG gene (Fig.3). There are two possible explanations. One is that the two mutations are really located on the LHβ gene of the patient and the other is that the CG sequence was amplified. However, the latter possibility is very



(2)

(3)

		986							1008
Wild	hLHβ	TGG	TGC	CAC	CCC	ATC	AAT	GCC	ATC
Patient	hLHβ	CGG							ACC
Wild	hCGβ	CGG		CGG					ACC

		8							15
Wild	hLHβ	TRP	CYS	HIS	PRO	ILE	ASN	ALA	ILE
Patient	hLHβ	ARG							THR
Wild	hCGβ	ARG		ARG					THR

Fig. 2. Comparison of DNA sequencing ladders of exon 2 of the LH-β subunit gene obtained from clones derived from proband (P) and a normal control (N). Arrows show the C→T substitution that occurs in codon 8 and 15.

Fig.3. Nucleotide sequence and predicted amino acid sequence of exon 2 of mutant LH-β subunit gene compared to codons 8-15 of the wild-type sequence of LH-β subunit gene and CG-β subunit gene. A T transits to C at the position of codons 8 and 15. These substitutions convert a TGG (Trp) to a CGG (Arg) codon and a ATC (Ile) to ACC (Thr) codon.

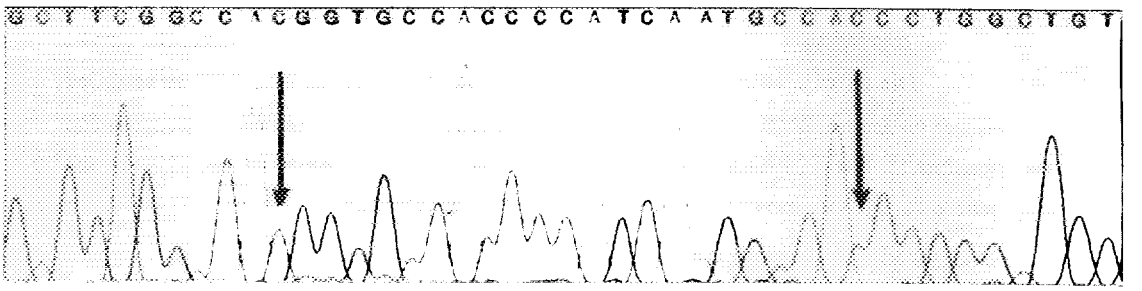
unlikely, because 1) these substitutions could be confirmed in all 10 clones isolated from the patient, whereas, all of the 10 clones from the PCR products of control DNA showed a normal LH sequence, and 2) the LH-type polymorphism, CAC(His) but not the CG-type polymorphism CGG(Arg) located at the 10th codon could be seen in all PCR product amplified from the patient. These findings lead us to conclude that the PCR product was derived from the LH β gene, but not from the CG β gene and that these two substituted amino acids cause the abnormality of the antigenicity of an anomalous LH. Also, the data that the two mutations are specific for the CG sequence and the rest of the amino acid residues are the same as those of LH, is in agreement with the clinical data that this anomalous LH showed normal bioactivity.

Thus, the anomalous LH β subunit showed the CG-type amino acid sequence at the 8th and 15th codon, but the rest of the sequence was identical to the LH sequence. Judging from the evolutional standpoint, this gene might be generated by the alternative evolutional diversion from a common ancestor gene of the gonadotropin family.

Pedigree analysis of the inherited anomalous LH

To confirm whether or not the LH β gene is really responsible for the phenotype and whether or not the two single base substitutions reflect the change of the antigenicity of the LH, we analyzed the patient with an anomalous LH, her parents, her brother and sister by PCR direct sequence. Figure 4a shows the direct sequencing pattern of the homozygote and heterozygote of the mutated LH β gene. The arrows show the positions of the mutated nucleotide. In these patterns, a single high peak is found in the homozygous

Homo



Hetero

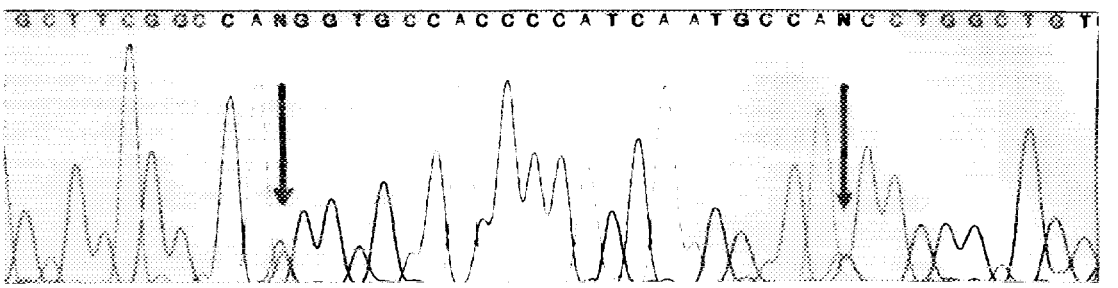


Fig.4a. The direct sequencing patterns of the homozygote and heterozygote of the mutated LH β gene were shown. The arrows show the positions of the mutated nucleotide. In these patterns, a single high peak is found in the homozygous pattern but two peaks, half as high as two different nucleotide signals are seen at the same position in the hetrozygous pattern.

pattern but two peaks, half as high as two different nucleotide signals are seen at the same position in the heterozygote pattern. These sequencing data are consistent with the sequencing data of the DNA clone isolated from the PCR products of the patient's DNA

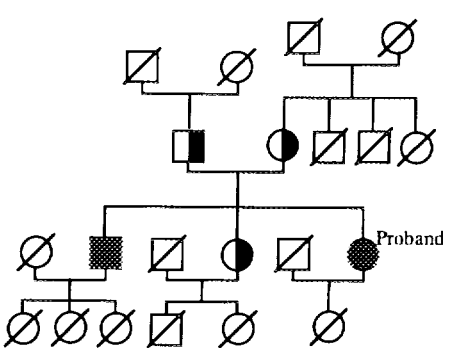


Fig.4b. Pedigree analysis of the patient for the mutation of LH. The parents and the sister of the patient were heterozygotes and the patient and her brother were homozygotes for the mutation, indicating that this trait follows Mendelian transmission.

and normal control. Thus, the homozygote and heterozygote for the mutation can be distinguished very clearly.

Next, we applied this method to pedigree analysis for the mutation. Figure 4b shows the results. The parents and the sister of the patient were heterozygote and the patient and her brother were homozygote for the mutation, indicating that this trait follows Mendelian transmission. These findings indicate that these two mutations in the LH β are responsible for the variance of the antigenicity in the patient's LH.

REFERENCES

1. Pierce,J.G. and Parsons,T.F.(1981) Annu.Rev.Biochem.150:465-495.
2. DecouVoutetakis,C.,Feltquate,D.M.,Drakopoufou,M.,Kouridas,I.A. and Dracopoll,N.C.
(1990) Am. J. hum. Genet. 48, 988-993.
3. Weiss,J.,Axelrod,L.,Whitcomb,R.W.,Crowley,W.F. and Jameson,J.L. (1992) New Engl. J. Med. 326, 179-183.
4. Matthews,C.H.,Borgato,S.,Beck-Pecoz,P.,Adams,M.,Tone,Y.,Gambino,G.,Casagrande, S.,Tedeschini,G., Benedetti,A. and Chatterjee,V.K.K. (1993) Nature genetics 5, 83-86.
5. Hayashizaki,Y.,Hiraoka,Y.,Endo,Y. and Matsubara,K. (1989) EMBO J. 8, 2291-2296.
6. Pettersson,K.,Ding,Y.-Q. and Huhtaniemi,I. (1992) J Clin Endocrinol Metab 74: 164-171.
7. Okuda,K.,Takamatsu,J.,Okazaki,T.,Yamada,T., Saeki,S. and Sugimoto,O., Endocrine Journal (in press).
8. Gross-Bellerd,M.,Oudet,P. and Chambon,P. (1973) Eur.J. Biochem.,36:32-38.
9. Sambrook,J.,Fritsch,E.F. and maniatis,T. (1989) Molecular cloning : recovery of DNA from low-melting-temperature agalose gels, p.6.30 Cold Spring Harbor Laboratory Press,USA.
10.Catt,K.J. and Pierce,J.G. (1974) In: Yen,S.S.C. and Jaffe,R.B.R.(eds) Reproductive endocrinology. W.B. Sauders Company, Philadelphia. vol 2: 34-62.
11.Fiddes,J.C. and Goodman,H.M. (1979) Nature, 281: 351-356.
12.Boorstein,W.R.,Vamvakopoulos,N.C. and Fiddes,J.C. (1982) Nature,300:419-422.
13.Otani,T.,Otani,F.,Krych,M.,Chaplin,D.D. and Boime,I. (1988)
J.Bio.Chem.263:7322-7329.