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Genetic Studies to Characterize the Origin of the Mutation in Placental Aromatase Deficiency

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Summary

Placental aromatase deficiency has recently been shown to be due to expression of RNA transcripts encoding abnormal aromatase molecules with 29 extra amino acids. To establish whether this aromatase deficiency is a hereditary or sporadic disease, we examined the genetic defect of the aromatase gene in the family of a patient. Direct sequencing of fragments of the aromatase gene prepared by PCR revealed that the splicing donor sequence (GT) of intron 6 in controls was mutated to GC in the patient, whereas the parents showed signals of both GT and GC. Subcloning of PCR products of the parents gave two different types of clones with GT and GC sequences in this site. Furthermore, for diagnosis of this deficiency, competitive-oligonucleotide-priming PCR of genomic DNA was performed in the presence of both normal and mutational oligonucleotide primers labeled with two kinds of fluorescent dyes, and the products were separated by agarose gel electrophoresis and were detected fluorometrically in the gel. Genomic DNA of the patient gave a PCR product primed only by the mutational primer, whereas that of controls gave a product primed only by the normal primer. The PCR products of the parents were primed by both primers. The results obtained by this fluorometric method were also confirmed by differential hybridizations with specific oligonucleotide probes. Thus these findings indicate that this deficiency is an autosomal hereditary disease and that the patient is a homozygote, while the parents are heterozygotes, for this mutation.

Introduction

Aromatase (estrogen synthetase; E.C.1.14.14.1) catalyzes the conversion of androgens to estrogens, which is a key step in estrogen biosynthesis, and constitutes a unique gene family (CYP19 gene) in the cytochrome P-450 superfamily (Nebert et al. 1989). This enzyme is localized in the ovary and placenta and plays an important role in the control of reproductive functions, by regulating the production of estrogens.

Recently, aromatase activity has also been observed in various extragonadal tissues, such as muscle (Loncope et al. 1978), liver (Smuk and Schwers 1977), hair follicles (Schweikert et al. 1975), adipose tissue (Schindler et al. 1972; Mendelson et al. 1982), and brain (Naftolin et al. 1972; Ryan et al. 1972). The expressions of aromatase in these tissues are regulated by various kinds of factors depending on the tissue. Furthermore, estrogens produced by this enzyme are reported to participate in sexual differentiation of the brain (MacLusky and Naftolin 1981), stimulation of bone formation (Takano-Yamamoto and Rodan 1990), and growth of hormone-dependent endometrial or breast cancer cells (Siiteri et al. 1976). Thus local estrogens in various extragonadal tissues seem to act as types of growth or differentiation factors and to have effects of a paracrine or autocrine type as well as having an endocrine action. These findings suggest that aromatase has essential roles for life, through estrogen production. There have been hardly any reports of definite cases of deficiencies of aromatase and estrogen receptor, whereas deficiency of androgen receptor has often been reported as testicular feminization syndrome (Lyon and Hawkes 1970; Keenan et al. 1974).

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Mango et al. (1978) first reported a clinical case of primigravida showing low urinary estrogen excretion and undetectable aromatase activity in the placenta. Recently, Shozu et al. (1991) observed clinical manifestations of progressive virilization of a primigravida, as well as female pseudohermaphroditism of her baby, and showed that they were caused by deficiency of placental aromatase activity. Further extensive studies showed that the aromatase gene of the patient was transcribed as a larger mRNA with an 87-bp insertion and was translated as a larger protein molecule with 29 extra amino acids, resulting in an almost inactive enzyme (Harada et al. 1992). Detailed sequence analysis of the patient's aromatase mRNA suggested that this placental aromatase deficiency was caused by abnormal splicing of aromatase transcripts that was due to a point mutation at the splice site.

It seemed possible that the genetic defect in the aromatase gene of the infant patient may be hereditary, since the parents are consanguineous in their pedigree (fifth degree). Therefore, in this work we performed DNA sequence analyses of the aromatase genes of the family of the patient, to establish whether this deficiency is a hereditary disease or was acquired by a sporadic gene mutation. We also developed a fluorometric method for gene diagnosis by a competitive-oligonucleotide-priming PCR (COP-PCR) with fluorescent primers, by which many samples can be examined within 1 d.

Material and Methods

Preparation of Human Genomic DNA

Peripheral blood samples (0.5–1.0 ml) were collected from healthy scientific personnel (from Aichi, Japan) and from the infant patient and the parents (from Kanazawa, Japan), with informed consent. The nuclear DNA fractions were obtained from blood lysates (Higuchi 1989) and were used directly as genomic DNA in the PCR.

Preparation of Oligonucleotide Primers and Probes

The oligonucleotides as shown in figure 1 were synthesized. H-AR-5 and H-AR-6 were used for PCR amplification; H-AR-N and H-AR-K were used for hybridization analysis; and H-AR-6N and H-AR-6K were used for COP-PCR. H-AR-5SQ contained a M13 sequencing primer sequence (5′ TGTAAAACGACGGCCAGT 3′) at the 5′ terminus of H-AR-5 and was used for direct sequencing of the aromatase gene.

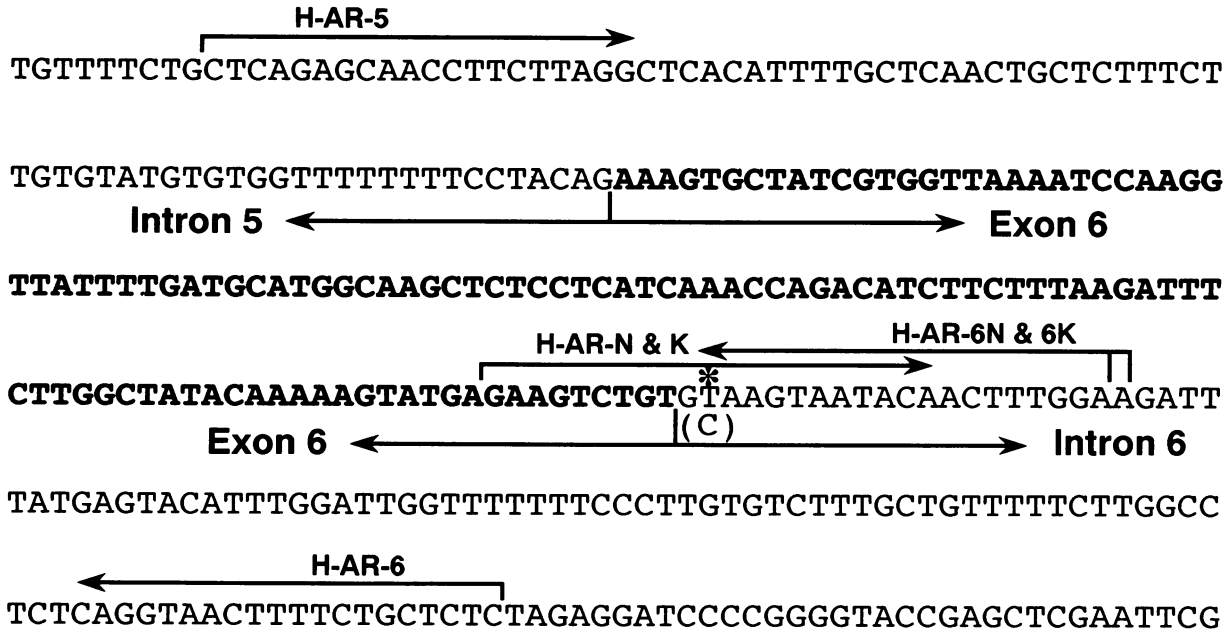

Figure 1 DNA sequence in the boundary region of exon 6 of the human aromatase gene, and sites of oligonucleotide probes and primers. The DNA sequences of exon 6 and of introns 5 and 6 are denoted by boldface and lightface letters, respectively. The asterisk (*) indicates a point-mutation site deduced from the patient's aromatase mRNA, and the mutated DNA sequence is shown in parentheses. The sequences of probes and primers are indicated by arrows.

H-AR-6N and H-AR-6K were attached by an aminohexyl linker (Aminolink-2; Applied Biosystems) to the 5′ termini on a DNA synthesizer 380B (Applied Biosystems) and then conjugated with the N-hydroxy succinimide ester derivatives of fluorescent dyes, 4′,5′-dichloro-2′,7′-dimethoxy-6-carboxyfluorescein (JOE; Applied Biosystems) and 5′-carboxyfluorescein (FAM; Applied Biosystems), respectively (Chehab and Kan 1989). The dye-labeled oligonucleotides were purified with a Sephadex G-25 column (PD-10; Pharmacia-LKB) and then by a reverse-phase cartridge (OPC; Applied Biosystems), according to the manufacturer's instructions.

Direct Sequencing of the Aromatase Gene

The aromatase gene fragments were amplified by the PCR (35 cycles) in the presence of H-AR-5SQ and H-AR-6 as primers. Amplified DNA fragments were purified by silica matrix and were sequenced directly by the dideoxy-chain termination method (Sanger et al. 1975) on a DNA sequencer 373A (Applied Biosystems). Simultaneously, the PCR products with H-AR-5 and H-AR-6 as primers were subcloned into Bluescript (Stratagene). Sufficient clones were sequenced to elucidate the illegible signals of the mutation site in sequencing of the heterozygote gene.

Hybridization Analysis with Oligonucleotide Probes

The DNA fragment containing the splicing donor site of intron 6 in the aromatase gene was amplified from genomic DNA by the PCR with the two primers H-AR-5 and H-AR-6 as described above and was loaded on 2.0% agarose gel. After electrophoresis, Southern blotting analysis was carried out (Maniatis et al. 1982). The blotted membrane was hybridized with ^{32}P-labeled H-AR-N and H-AR-K. Hybridization was performed at 50°C for 18 h in 5 × SSC, 1 × Denhardt's solution, 0.2% SDS, and 100 μg sheared salmon sperm DNA/ml. After the membrane was washed as specified in the figure legends, an autoradiogram was prepared.

COP-PCR Analysis with Fluorescent Primers

In this COP-PCR analysis, JOE-labeled H-AR-6N (25 pmol) and FAM-labeled H-AR-6K (25 pmol) were used as competitive reverse primers, instead of the single reverse primer H-AR-6. The three primers in the PCR were H-AR-5 upstream primer, specific for both the normal and the patient's aromatase gene, and H-AR-6N and H-AR-6K downstream primers, specific for the normal and the patient's aromatase gene, respectively. The PCR was performed for 25 cycles as follows: 94°C for 40 s, 53°C for 1 min, and 72°C for 30 s. PCR products (4 μl) were mixed with 3 μl of fluorescent size standard, GENESCAN-1000ROX (Applied Biosystems), and were loaded on 2% SeaPlaque GTG agarose (FMC BioProducts) gel. During electrophoresis of the PCR products, fluorometric detection was performed by laser scanning with a Gene Scanner 362 Fluorescent Fragment Analyzer (Applied Biosystems).

Results and Discussion

Direct Sequencing of the Mutation Region in the Aromatase Gene from the Patient and the Parents

A previous study of human placental aromatase deficiency revealed that aromatase mRNA of the patient's placenta had an extra 87-bp insertion in the coding region and suggested that this insertion was caused by abnormal splicing due to a point mutation in the splicing donor sequence of the aromatase gene (Harada et al. 1992). For determination of whether the point mutation in the patient's aromatase gene was sporadic or hereditary, direct sequencing of the mutation region of the patient and the parents was carried out as shown in figure 2, after PCR amplification of this region. The splicing donor sequence in intron 6 of the normal aromatase gene was ascertained to be GT according to the canonical GT/AG rule (Mount 1982), whereas this site in the patient's aromatase gene was changed to GC. Furthermore, both the mother and the father of the patient had illegible signals of both T and C after G in the corresponding region of the aromatase gene (figs. 1 and 2), although in both cases the sequence of this site was read as C by the DNA sequencer.

For confirmation that both parents are heterozygous for the point mutation in the aromatase gene, DNA fragments of their mutation region were amplified by the PCR, were subcloned into Bluescript, and were sequenced. When 16 independent clones each from fragments of the father and mother were sequenced, 5 and 7 clones were found to have definite GT sequences, and 11 and 9 clones were found to have definite GC sequences, in this region of the aromatase gene (data not shown). These results indicate that the patient is homozygous and that both parents are heterozygous for this genetic mutation—and thus that this aromatase deficiency is a hereditary disease with an autosomal recessive trait.

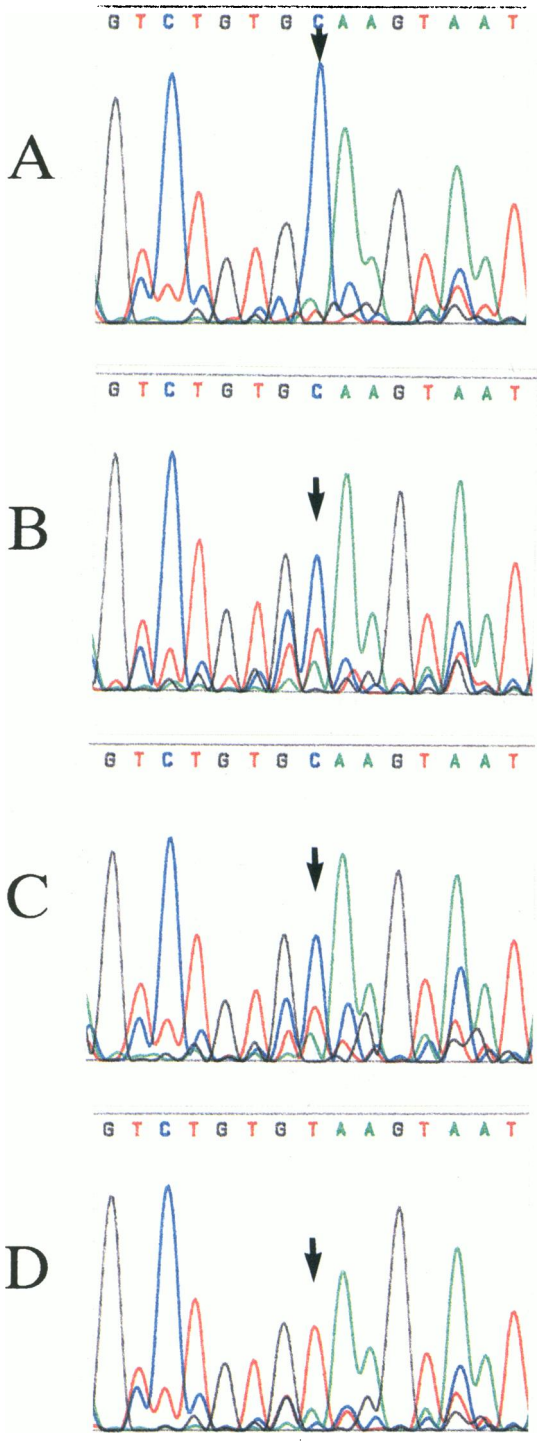

Figure 2 Direct sequencing of the aromatase gene amplified by the PCR. DNA fragments from the aromatase genes of the patient, the parents, and controls were prepared by PCR amplification of the genomic DNAs. The PCR products were directly sequenced with a DNA sequencer. *A*, Patient's gene. *B*, Mother's gene. *C*, Father's gene. *D*, Control's gene. The site of one point mutation in this deficiency is indicated by an arrow in each sequence.

Hybridization Analysis of Aromatase Genes by Allele-specific Oligonucleotides

Further confirmation of the above results was obtained by hybridization analysis of the aromatase gene by allele-specific oligonucleotide probes (fig. 1). These probes were designed to have the mutation site in the middle of allele-specific 21-long oligonucleotides, to maximize the thermal instability of mismatched hybrids (Kidd et al. 1983). The mutation region of the aromatase gene of the patient and both parents was amplified by the PCR, was blotted on a nylon membrane after agarose gel electrophoresis, and hybridized with ^{32}P-labeled H-AR-N or H-AR-K oligonucleotide probes, which are specific for the normal and mutation sequence, respectively. After hybridization at 50°C, washing at 50°C removed most nonspecific binding, including the binding of a primer with a single-base mismatch, from the membrane as shown in figure 3. Consequently, H-AR-N hybridized strongly with the normal aromatase gene and significantly with those of both parents, but not with the patient's gene. On the contrary, H-AR-K hybridized strongly with the pa-

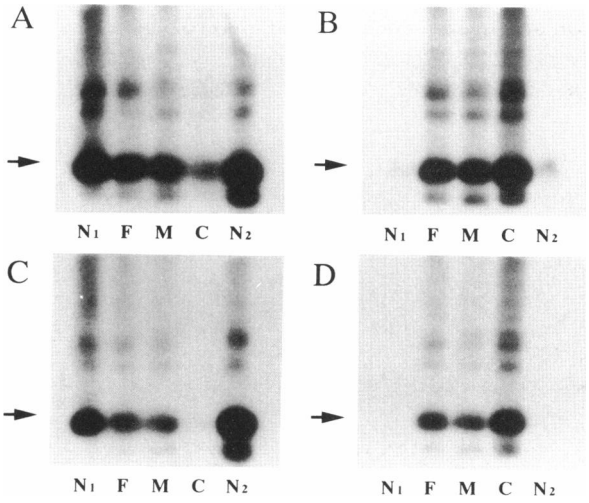

Figure 3 Hybridization analysis by allele-specific oligonucleotide probes. The DNA fragments containing the mutation site were amplified from genomic DNAs by the PCR and were subjected to agarose gel electrophoresis followed by Southern blotting analysis. The blotted membranes were hybridized with one of two ^{32}P-labeled oligonucleotide probes, H-AR-N (*A* and *C*) and H-AR-K (*B* and *D*), at 50°C for 18 h and were washed thoroughly with 2 × SSC and then with 0.2 × SSC containing 0.1% SDS at either 25°C (*A* and *B*) or 50°C (*C* and *D*). The arrow in each autoradiogram indicates the estimated position of an aromatase gene fragment amplified by the PCR. Lanes N_1 and Lanes N_2, Control's gene. Lanes F, Father's gene. Lanes M, Mother's gene. Lanes C, Patient's gene.

tient's aromatase gene and significantly with those of both parents, but not with the normal gene. On the other hand, washing at 25°C permitted both probes to remain bound to the aromatase gene in all cases, although the binding of primers with a single-base mismatch to the corresponding gene was weak. In particular, the binding of H-AR-N with a single-base mismatch between G and T was firmer than that of H-AR-K with a single-base mismatch between A and C, as shown in figure 3. These results also support the results of DNA sequencing of the aromatase genes of the patient and the parents.

Further Screening of Aromatase Genes by COP-PCR with Fluorescent Primers

To screen for this deficiency in more normal controls, we developed a convenient fluorometric assay by a COP-PCR technique. COP-PCR was originally developed by Gibbs et al. (1989) to detect a single-base mutation in murine ornithine transcarbamylase deficiency by using radiolabeled oligonucleotide primers. This technique has also been used to detect a single-base change in familial gene analysis of human hypoxanthine phosphoribosyltransferase deficiency (Gibbs et al. 1989). Recently, a color-complementation assay has been reported to detect specific DNA sequences by fluorescence PCR amplification (Chehab and Kan 1989). We developed the method of COP-PCR with fluorescent primers, which combines the merits of both analytical procedures. This method is easier, faster, and more quantitative than previous methods and also does not require radioactive materials, so it is convenient for use in a clinical laboratory for mass screening of DNA for genetic diseases. Furthermore, the fluorescent dye–labeled primer can be stored at −80°C for a long time, and so DNA samples can be analyzed promptly whenever necessary. In the case of COP-PCR analysis of aromatase deficiency, we can usually analyze 24 peripheral blood samples with a Gene Scanner within 12 h.

The competitive primers H-AR-6N and H-AR-6K were designed to have the point mutation at the 3′ end

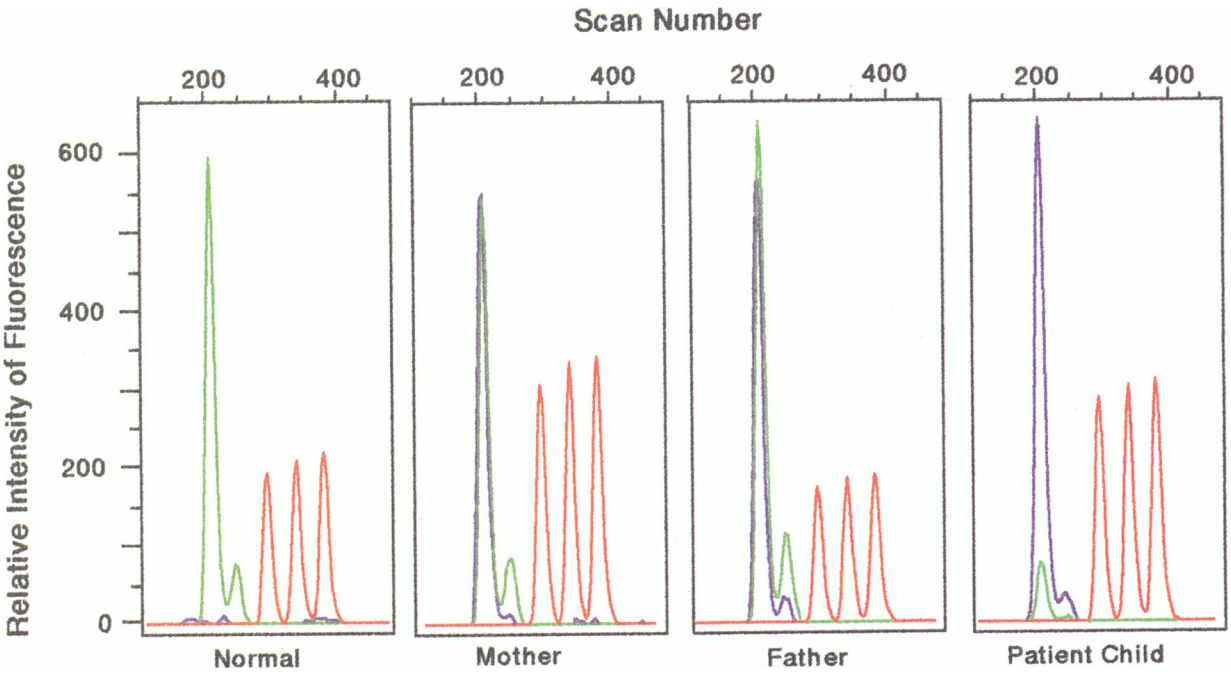

Figure 4 Genetic analysis of aromatase deficiency, by COP-PCR with fluorescent primers. The aromatase gene fragment containing the mutation region was amplified from a genomic DNA by COP-PCR with H-AR-5 as an upstream primer and with both JOE-labeled H-AR-6N and FAM-labeled H-AR-6K as competitive downstream primers. PCR products were analyzed fluorometrically with a Gene Scanner 362 (Applied Biosystems) during electrophoresis in agarose gel. Red lines denote an internal size standard, which consists of fragments of 262, 293, and 317 bp. PCR products primed with JOE-labeled H-AR-6N and FAM-labeled H-AR-6K are denoted by green and blue lines, respectively. The major PCR product in each assay is 210 bp, corresponding to the calculated sizes of 210 bp for H-AR-6K and 211 bp for H-AR-6N.

of the primer sequence (fig. 1) and were labeled with two kinds of fluorescent dyes at their 5′ end. To correct for the difference in melting temperature of the two primers, H-AR-6N having A at the 3′ end and H-AR-6K having G at the 3′ end were 20 and 19 bases long, respectively.

The mutation regions of aromatase genes from the patient, the parents, and controls were amplified by the PCR in the presence of competitive primers labeled with the fluorescent dyes FAM and JOE. The PCR product from controls was labeled only with JOE, not with FAM, whereas that from the patient was labeled with FAM, but little with JOE, as shown in figure 4. The PCR products of both parents gave almost equal peaks of fluorescence with JOE and FAM, on analysis with the Gene Scanner. These results clearly indicate that the placental aromatase deficiency in this patient is a hereditary disease with an autosomal recessive character and that this technique of COP-PCR with fluorescent primers is useful for DNA diagnosis of this deficiency.

We have been screening the scientific personnel of this institute for this deficiency, by using this new fluorometric technique of COP-PCR. So far we have examined samples from 47 volunteers and have found that they are all homozygous for the normal aromatase gene. We have reported that this mutation causes severe loss of aromatase activity, as judged on the basis of the transient expression of the mutant aromatase cDNA in COS-7 cells. These results show that the mutation is not merely a human genetic polymorphism but is also the true cause of this hereditary aromatase deficiency disease.

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