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ORIGINAL PAPER

Gonadotropin-independent precocious puberty associated with a somatic activating mutation of the LH receptor gene: detection of a mutation present in only a small fraction of cells from testicular tissue using wild-type blocking polymerase chain reaction and laser-capture microdissection

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Abstract *Objective* Leydig cells are the principal source of testosterone, and boys with Leydig cell tumors typically have signs of gonadotropin-independent precocious puberty as a result of testosterone secretion by the tumor. A single somatic activating mutation of the LH receptor gene, Asp578His, limited to the tumoral Leydig cells, has been described in a few boys with gonadotropin-independent precocious puberty. We report a molecular study of a boy with gonadotropin-independent precocious puberty caused by a Leydig cell tumor. *Design and setting* This is a clinical case report from the Kobe Children's Hospital. *Patient and methods* One patient with gonadotropin-independent precocious puberty caused by a Leydig cell tumor underwent a left orchidectomy. We performed a genetic study of the tumoral Leydig cells. *Result* Using wild-type blocking PCR (WTB-PCR) and laser-capture microdissection (LCM), we found that the Asp578His mutation of the LH receptor gene was exclusively localized to the tumoral Leydig cells and was absent in the adjacent normal tissue and leukocytes. *Conclusions* WTB-PCR and LCM are powerful techniques that can detect a somatic mutation present in only a small fraction of cells from heterozygous tissue samples.

Keywords Precocious puberty · Leydig cell tumor · LH receptor · Wild-type blocking PCR · Laser-capture microdissection

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Introduction

Testicular sex-cord stromal tumors (SCSTs) show differentiation toward Leydig cells, Sertoli cells, and/or other types of sex cord-stromal cells. They are much less common than germ cell tumors, and account for less than 5% of all testicular neoplasms in adults. Testicular SCSTs are somewhat more common in prepubertal males. Leydig cell tumors are the most common type of testicular SCST, and may occur in any age group, including young children. The peak incidences are at ages 5–10 and 30–40 years [1, 2]. Up to 20% of Leydig cell tumors in adults are malignant, but malignant behavior has not been documented in children [3, 4]. Leydig cells are the principal source of testosterone, and boys with Leydig cell tumors typically have signs of gonadotropin-independent precocious puberty as a result of testosterone secretion by the tumor. A single somatic activating mutation of the LH receptor gene, Asp578His, limited to tumor cells, has been described in a few boys with gonadotropin-independent precocious puberty [5–9]. Here, we describe a molecular study of a boy with gonadotropin-independent precocious puberty caused by a Leydig cell tumor. In our study, we used wild-type blocking PCR (WTB-PCR) and laser-capture microdissection (LCM), which are powerful

tools that can be used to detect mutations present in tumor cells.

Patient and methods

Patient

The patient, a Japanese male, was first seen at Kobe Children's Hospital at the age of 3 years, 11 months, to evaluate the enlargement of his penis. Family history of precocious puberty was negative. Early growth and development seemed normal. However, by the time he was 2 years, 6 months old, his mother became aware of the enlargement of his penis. At the age of 3 years, 11 months, the patient's height was 2.0 SD above the Japanese mean height for chronological age. His bone age was 6 years. He had an enlarged phallus (7.0 cm in length) with a corrugated scrotum containing testes measuring 12 × 24 mm bilaterally, and pubic hair corresponding to Tanner stage 2. No testicular mass was palpable. Ultrasound examination of the testis found no tumor. Color-coded sonography was not performed at that time. Hormonal evaluation revealed gonadotropin-independent testosterone hypersecretion (serum testosterone, 314 ng/dl; basal serum LH, <0.05 IU/l; basal serum FSH, 0.18 IU/l; after GnRH stimulation: maximum LH, 0.64 IU/l; maximum FSH, 3.87 IU/l). Hormonal evaluation and bone scintigraphy ruled out the diagnosis of congenital adrenal hyperplasia, a chorionic gonadotropin secreting tumor, and McCune–Albright syndrome. Although the clinical features of the patient were compatible with the diagnosis of familial male-limited precocious puberty resulting from activating germline mutation in the LH receptor gene, no mutation was identified in exon 11 of the LH receptor gene when DNA isolated from blood was analyzed.

Therapy with bicalutamide and anastrozole was initiated at the age of 4 years. 1 month. After 12 months of treatment, a partial reduction in growth rate was observed (before treatment, 17.9 cm/year; after treatment, 9.3 cm/year), but the treatment did not result in normalization of growth rate or reduction in rate of bone age maturation. The testes volume increased asymmetrically (left side, 13 × 30 mm; right side, 12 × 24 mm). Ultrasound examination revealed a focal hypoechoic area in the left testis, and color-coded sonography showed a focal hyperperfusion within the hypoechoic area. A left orchiectomy was performed at the age of 5 years, 2 months. After the orchiectomy, his serum testosterone reverted to prepubertal levels (<5.0 ng/dl). Histological examination showed that the tumor was non-capsulated, poorly circumscribed, and composed of nests of polygonal Leydig cells with abundant eosinophilic cytoplasm and centered ovoid nuclei.

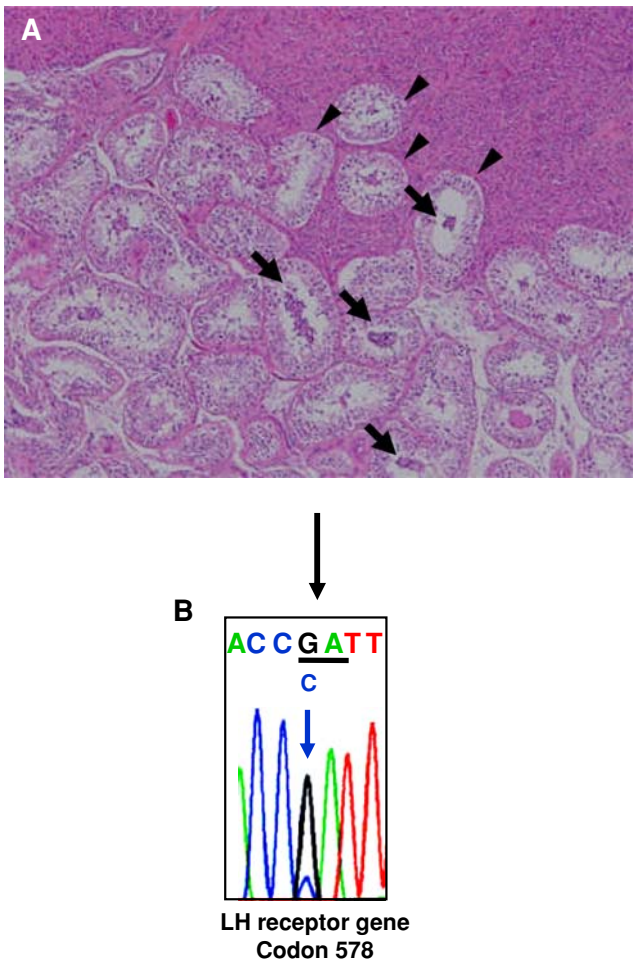

Fig. 1 Histology and DNA analysis of the Leydig cell tumor. **a** Histological examination revealed that the tumor was non-capsulated, poorly circumscribed, and composed of nests of polygonal Leydig cells with abundant eosinophilic cytoplasm and centered ovoid nuclei (upper right part of the figure). Spermatogenesis was identified in the seminiferous tubules (*arrows*). Some seminiferous tubules were surrounded by tumor cells (*arrowheads*). **b** Direct sequencing of exon 11 of the LH receptor gene from the frozen tumor tissue. The chromatograph shows the genomic DNA sequence of codon 578 of the LH receptor gene

Spermatogenesis was identified in the seminiferous tubules (arrows). Some seminiferous tubules were surrounded by tumor cells (arrowheads) (Fig. 1a).

Methods

Amplification and sequencing of DNA

The patient's parents gave their written consent for the necessary surgery, tumor removal, and analysis of the tumor tissue.

Genomic DNA was extracted using a standard method (Qiagen, Valencia, CA, USA) from the frozen tumor tissue

and from frozen normal tissue that was adjacent to the tumor. The 11 coding exons and exon–intron boundaries of the LH receptor gene were amplified by PCR and automatically sequenced.

Wild-type blocking PCR (WTB-PCR)

To verify the presence of the mutation at codon 578 in the tumor tissues, we performed WTB-PCR [10] on genomic DNA extracted from the frozen tumor tissue samples. We designed an LNA-substituted blocking oligonucleotide to inhibit amplification of the wild-type exon 11 of the LH receptor gene, while still permitting amplification of the mutant exon 11. The blocking nucleotide was made homologous to a region of the wild-type exon 11. Three mismatched bases were added to its 3′ terminus to prevent extension by DNA polymerase. The following sequence was used for the blocking oligonucleotide (LNA bases underlined): 5′-CTTCACCGATGGA-3′. The following primers were used for amplification of the mutant exon 11: forward, 5′-TGGCAATCCTCATCTTCACC-3′ and reverse, 5′-TGAAGCCATTTTTGCAGTTG-3′. Each 50 µl reaction contained 18.5 µl of distilled water, 25 µl of HotStar Taq Master Mix (Qiagen, Valencia, CA, USA), 0.75 µl of each primer (0.15 pmol/µl), 4 µl of LNA blocking oligonucleotide (8 pmol/µl), and 1 µl of DNA template. PCR conditions were as follows: 15 min at 95°C, 40 cycles of 30 s at 95°C, 60 s at 68°C, 30 s at 60°C, and 45 s at 72°C, with a final extension of 5 min at 72°C (Fig. 2).

Laser-capture microdissection (LCM)

To confirm the presence of the mutation in the Leydig cells, LCM of the paraffin embedded tissue was performed using a PixCell II (Arcturus Engineering, Inc., Mountain View, CA, USA) [11]. The transfer film with attached specimen was reverse mounted on a coverslip. The specimens were then subjected to a laser beam and the targeted cells (tumoral Leydig cells) were catapulted by laser pressure onto mineral oil-coated PCR tube caps. The genomic DNA was then extracted from the targeted cells. Exon 11 of the LH receptor gene was amplified by PCR and automatically sequenced.

Results

No mutation was identified in the LH receptor gene in DNA extracted from the normal tissue adjacent to the tumor. Genetic analysis of the frozen tumor tissue suggested a mutation from G to C in the first position of codon 578 of exon 11 of the LH receptor gene. This mutation

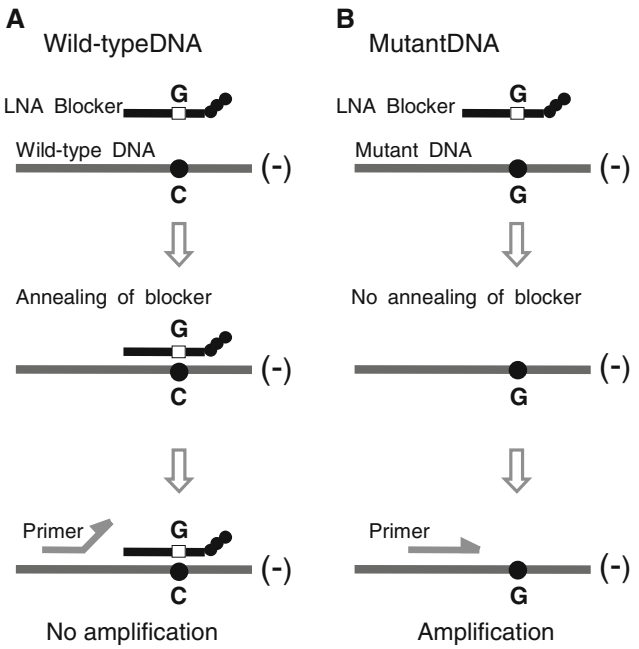

Fig. 2 Wild-type blocking PCR. **a** The LNA blocker sequence anneals with the wild-type sequence to prevent amplification of wild-type DNA. **b** The base pair mismatch between the LNA blocking sequence and the mutant DNA sequence prevents annealing of the blocking sequence. Therefore, polymerase chain reactions amplify only the mutant DNA sequence [10]

changed the encoded amino acid from asparaginic acid (GAT) to histidine (CAT). However, the peak height of the mutant nucleotide C was much lower than that of the wild-type nucleotide G on the sequence chromatogram (Fig. 1b). This finding suggests that the tissue sample of the tumor contained an excess of wild-type cells such as vascular endothelial cells, Sertoli cells, and germ cells.

To verify the presence of the mutant nucleotide C in the first position at codon 578, we performed WTB-PCR. We designed an LNA-substituted blocking oligonucleotide to inhibit amplification of the wild-type exon 11, while still permitting amplification of the mutant exon 11. PCR amplification of DNA from the frozen tumor tissue with the LNA blocker gave a clear, visible band corresponding to exon 11, whereas PCR amplification of DNA from blood with the LNA blocker gave no visible band. After WTB-PCR amplification with the LNA blocker, direct sequencing of exon 11 from the frozen tumor tissue clearly showed the presence of the Asp578His mutant sequence at codon 578 (Fig. 3).

Genetic analysis of the tumoral Leydig cells obtained by laser capture microdissection confirmed the heterozygous G to C mutation in the first position at codon 578 (Fig. 4). These findings indicate that the heterozygous Asp578His mutant sequence was limited to the tumoral Leydig cells and that the mutation was somatic.

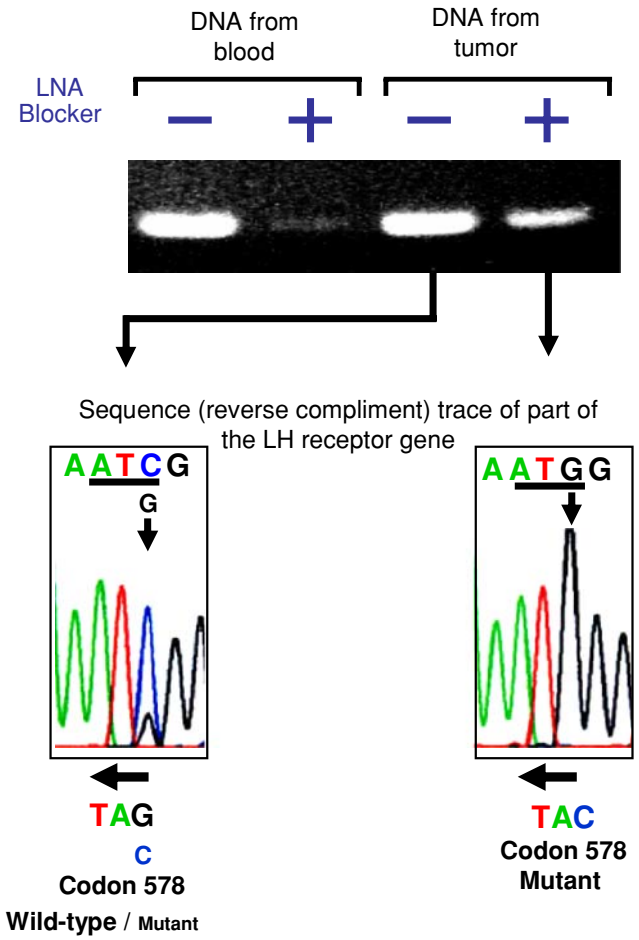

Fig. 3 Detection of the LH receptor gene mutation at codon 578 in DNA obtained from frozen tumor tissue using wild-type blocking PCR (WTB-PCR). Upper panel: WTB-PCR amplification of DNA from the frozen tumor tissue with the LNA blocker shows a clear visible band corresponding to exon 11, whereas WTB-PCR amplification of DNA from blood with the blocker gave no visible band. Lower panel: direct sequencing of the amplified product of WTB-PCR from the frozen tumor tissue with the LNA blocker clearly showed the presence of the Asp578His mutant sequence at codon 578

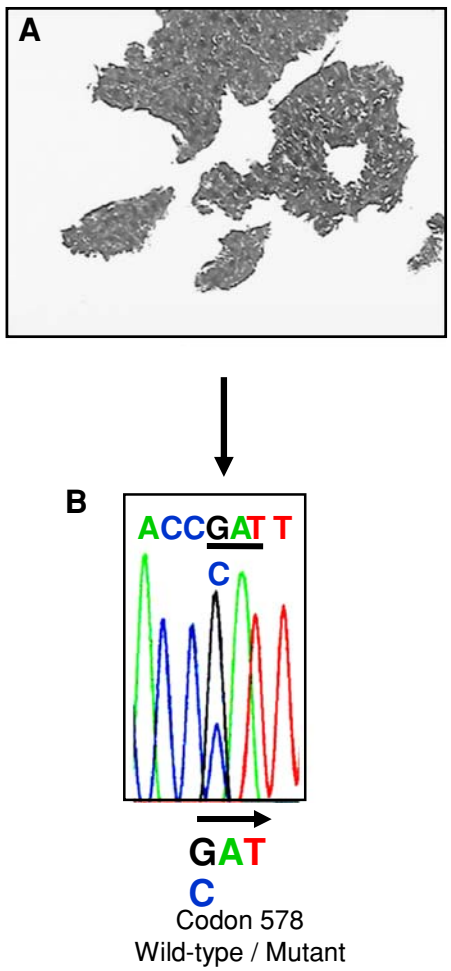

Fig. 4 Detection of the LH receptor gene mutation of codon 578 in DNA from the paraffin-embedded tumor tissue after laser-capture microdissection. **a** The target tumor cells were captured by laser-capture microdissection. **b** Direct sequencing of the PCR product from the tumor cells captured by laser-capture microdissection revealed the heterozygous G to C mutation in the first position at codon 578 in the tumoral Leydig cells

Discussion

Detection and sequencing of mutations from clinical specimens of tumor tissue is often complicated by the presence of admixed nonmutated cells. In our study, WTB-PCR and LCM provided powerful approaches that can be used to detect mutations present in only a small proportion of cells from heterozygous tissue samples. Using WTB-PCR and LCM, we confirmed that the Asp578His mutation of the LH receptor gene was exclusively localized to the tumoral Leydig cells and was absent in the adjacent normal tissue and leukocytes. These findings imply that the Leydig cell tumor has a specific monoclonal alteration in DNA and is derived from the successful proliferation of one initial cell in which the causal mutation of Asp578His of the LH receptor gene has occurred.

This single somatic Asp578His mutation of the LH receptor gene has been described in nine boys, including our case, with gonadotropin-independent precocious puberty caused by Leydig cell tumors [5–9]. In contrast, more than 15 germline mutations causing familial male-limited gonadotropin-independent precocious puberty have been reported [12]. These observations suggest that the Asp578His mutation contributes to both selective growth advantage and hormonal hyperfunction, whereas that the germline mutations causing familial male-limited precocious puberty alter hormonal function but confer no selective growth advantage. Liu et al. [5] reported that the somatic mutation found in the Leydig cell tumors of boys with precocious puberty (i.e., the Asp578His mutant) is unique in its ability to activate two signaling pathways (i.e., cAMP and inositol phosphate accumulation) and that it

differs from the germline activating mutations found in boys with precocious puberty (such as Asp578Tyr and Leu457Arg), which were reported by Liu et al. to activate only the cAMP pathway. They speculated that neoplastic transformation of Leydig cells with the Asp578His mutation involves inappropriate costimulation or synergism of the cAMP and phospholipase C pathways [5]. However, Hirakawa et al. [13] found that mutations causing familial male-limited precocious puberty were constitutively active not only on cAMP accumulation but also on inositol phosphate accumulation. Thus the mechanism by which the Asp578His mutation of the LH receptor gene results in tumor formation remains to be elucidated.

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