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An exon-skipping mutation in the *btk* gene of a patient with X-linked agammaglobulinemia and isolated growth hormone deficiency

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Abstract

X-linked agammaglobulinemia (XLA) is an inherited immunodeficiency disease associated with a block in differentiation from pre-B to B cells. The XLA gene encodes a 659 amino acids cytoplasmic protein tyrosine kinase named *btk* (Bruton's tyrosine kinase). The few *btk* gene alterations so far reported in XLA patients are heterogenous and distributed in all domains of the *btk* protein. They appear to be responsible for a range of B cell immunodeficiency disorders of variable severity. Rare families in which XLA is inherited together with isolated growth hormone deficiency (IGHD) have been reported. Genetic analysis has shown that this disease association maps to the same region of the X chromosome as XLA, but whether the two phenotypes are caused by a common or different developmental or biochemical mechanism is unknown. We have analysed the *btk* gene of a patient with XLA and IGHD. RT-PCR analysis of *btk* transcripts, sequencing data obtained from cDNA and genomic DNA and in vitro splicing assays showed that an intronic point mutation (1882 + 5G→A) is responsible for skipping of an exon located in the tyrosine kinase domain. This exon-skipping event results in a frameshift leading to a premature stop codon 14 amino acids downstream, and in the loss of the last 61 residues of the carboxy-terminal end of the protein. Although we studied a sporadic case, the results suggest that an alteration of the *btk* gene might cause this unusual phenotype.

Key words: X-linked agammaglobulinemia; Isolated growth hormone deficiency; *btk* gene; Exon skipping

1. Introduction

X-linked agammaglobulinemia (XLA) is an inherited immunodeficiency disease that affects males, who are generally identified by their unusual susceptibility to bacterial infections [1]. XLA patients have very low serum levels of all immunoglobulin classes, their plasma cells are virtually absent and B cells are almost undetectable in lymphoid tissues, where they mature, or in the blood stream [2,3]. However, the number of pre-B cells in bone marrow is normal [4] and T cells are apparently unaffected. The causative defect seems to prevent transition of pre-B cells into B cells. Female obligate carriers of XLA appear to be immunologically normal, but X chromosome inactivation is not random in B lymphocytes, probably because the B cell in which the XLA-bearing chromosome is active cannot mature [5–7]. This feature enables female carriers to be identified in affected families [5].

The XLA gene, which is contained in the mid portion of the long arm of the X chromosome at Xq21.3–Xq22 [8–13], has recently been isolated by positional cloning [14] and as a cDNA clone in a library derived from murine B-cell progenitors [15]. The cloned gene encodes a 659 amino acid cytoplasmic protein tyrosine kinase named *btk* (Bruton's tyrosine kinase) which is expressed in B lymphocytes and myeloid cells as well as in lung and pancreas, but not in the T cell lineage. Sequence analysis of *btk* has revealed homologies with the *Drosophila melanogaster* Dsrc28C kinase, *tec*I and *tec*II mammalian kinases, *itk* murine kinase [15] and the recently identified EMT human kinase expressed mainly in T lymphocytes and in natural killer cells [16]. All members of this new family of intracellular protein tyrosine kinases share the following characteristics: (1) a large unique amino terminal region lacking myristylation sites, (2) two *src* homology (SH) regulatory domains, SH2 and SH3, (3) a highly conserved tyrosine kinase domain containing an ATP-binding site and a substrate-specific domain, and (4) a short carboxy terminus lacking the negative regulatory tyrosine residue found in *src* family members (Fig. 1c).

The few *btk* gene alterations reported so far in XLA patients include small and large deletions, insertions, and a few missense and stop defects [14,17,18]. These mutations have been identified in all domains of the *btk* protein, and appear to be responsible for a range of B cell immunodeficiency disorders of variable severity.

There have been rare reports of families in which X-linked agammaglobulinemia is inherited together with isolated growth hormone deficiency (IGHD) [19–21]. Examination of X chromosome inactivation and genetic

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Abbreviations: btk, Bruton's tyrosine kinase; GH, growth hormone; IGHD, isolated growth hormone deficiency; PCR, polymerase chain reaction; RT, reverse transcription; XLA, X-linked agammaglobulinemia.

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linkage analysis in these families have shown that the disease association maps to the same region of the X chromosome as Bruton's disease [22]. It is not known whether the *btk* gene itself or if mutations in another gene located in the vicinity of the XLA locus account for this complexe phenotype. We analyzed the *btk* gene of a patient with both classical XLA and IGHD (peak hGH responses of <5 ng/ml hGH to various provocative tests). We found that an intronic point mutation causes skipping of an exon located in the kinase domain, and leads to truncation of the carboxyterminal part of the *btk* protein. These results suggest that alterations of the *btk* gene may cause the unusual mixed disease phenotype.

2. Materials and methods

2.1. Patient

The male patient belongs to a large family with no history of immunodeficiency or short stature. XLA was diagnosed at 2 years of age, when he was seen for recurrent bacterial infections. He had severe hypogammaglobulinemia, with IgM levels of 0.05 mg/ml (normal range 0.54–1.14 mg/ml), IgG of 0.08 mg/ml (6.1–10.7 mg/ml) and IgA of 0.05 mg/ml (0.46–1.1 mg/ml). He was treated with gammaglobulin infusions.

Patient growth was normal until 2 years of age, then progressively decreased to reach 122 cm (−3.2 SD) at 10.5 years of age. Physical examination, including genitalia, was normal. Ornithine and betaxolol-glucagon stimulation tests revealed complete GH deficiency with plasma GH < 1.7 ng/ml. Other pituitary functions, including TSH, PRL, ACTH and gonadotropins, were normal. These data establish the diagnosis of isolated severe somatotropic deficiency. The patient, who is now 17 years old, is > 3 SD below mean height (155 cm).

2.2. Nucleic acid purification, reverse transcription and polymerase chain reaction amplification

Total RNA and genomic DNA were purified from Epstein-Barr virus-transformed pre-B cells from the patient's bone marrow, and B cells from his mother's and a healthy control's peripheral blood, using guanidium isothiocyanate and proteinase K treatment, as described in [23]. cDNA copies of the *btk* RNA sequence were synthezised from 10 μg of total RNA in a 50 μl reaction mixture containing 10 pmol antisense primer P2 and AMV reverse transcriptase (Promega Laboratories, Madison, WI) in conditions recommended by the supplier. The cDNA and genomic DNA sequences were subjected to amplification essentially as described by Saiki et al. [24], with 30 cycles (denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 90 s at 72°C).

The sequences of the oligonucleotides used were deduced from the published sequence of *btk* cDNA [14] and numbered according to Vetrie et al. [14] except for P7 which was derived from the sequence of the 3 kb genomic DNA fragment cloned in this work.

The primers were as follows:

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P1:5'-GAAAGAAGAAGCTATGGCCGCAG-3'
P2:5'-TTGGCGAGCTCAGGATTCTTCATCCATG-3'
P3:5'-CTCCTGAACTACCTGAGGGAG-3'
P4:5'-TTCAGCAGTCTCACTG-3'
P5-B:5'-CGCGGATCCGCGGTTTGGTAAACGATCAAGGAG-3'
P6-B:5'-CGCGGATCCGCGTGTGTTCAGCAGTCTCACTG-3'
P7:5'-TCATCCTCAGAAACCCTGTG-3'
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2.3. Direct sequencing of PCR-amplified cDNA and genomic DNA

The sequences of cDNA and genomic DNA samples were determined after asymmetric amplification (50 pmol of one primer and 2 pmol of the other) as described elsewhere [25].

2.4. Cloning and sequencing of PCR products

PCR amplification was performed with oligonucleotides P3 and P4 on control genomic DNA. The product was subcloned into vector pCRII according to the supplier's instructions (TA cloning kit, InVitrogen) and sequenced using the dideoxy chain-termination method.

2.5. Site-directed mutagenesis and splicing assays

PCR amplification products were generated from control and patient genomic DNA using oligonuclotides P5-B and P6-B, containing a *Bam*HI site in their 5′ ends. After *Bam*HI digestion, they were subcloned into the *Bam*HI sites of vector pSPL1 (Exon trapping kit, BRL). The PCR fragment obtained from the patient's genomic DNA was subcloned into vector pCRII and subjected to site-directed mutagenesis using the Transformer site-directed mutagenesis kit (Clontech Laboratories) with oligonucleotides P-1882 + 5G (5′-GGGCTTTTGGTAAGTGGATAAGATTACACAG-3′) and pCRII-1880*NcoI*/*ClaI* (5′-CTCGTCGTGATCGATGGCGATGCC-3′). After mutagenesis, the *Bam*HI-digested PCR fragment was subcloned into pSPL1.

Eighty-percent-confluent COS-7 cells were transfected by the lipofectine method (Gibco/BRL Life Technology, Grand Island, NY) with 2 μg of each pSPL1 recombinant. Approximately 48 h after transfection, total RNA was extracted [23] reverse transcribed and PCR-amplified as described above, with oligonucleotides SD3 and SA4 supplied with the kit (Exon trapping kit, BRL).

3. Results

3.1. An interstitial deletion in btk *mRNA*

The *btk* gene was analysed in cDNAs prepared from RNA extracted from Epstein-Barr virus (EBV)-transformed pre-B cell lines from the patient and EBV-transformed B cells lines from his mother and a healthy control. In these three individuals, amplification of the cDNAs with primers P1 and P2 generated PCR products spanning the whole *btk* coding region (approximately 2 kb). Overlapping segments of the coding region were amplified to assess the integrity of the *btk* transcript in the patient. The amplification product obtained with oligonucleotides P3 and P4 was shorter in the patient (262 bp) than in his mother and the control (381 bp) (Fig. 1a). Sequence analysis of these fragments revealed that the shorter transcript lacked nucleotides 1764 to 1882, numbered according to Vetrie et al. [14] (Fig. 1b). This deletion introduces a frameshift that changes 14 amino acids and creates a premature stop codon which would lead to the loss of the last 61 residues of the carboxy-terminal end of the protein (Fig. 1c).

3.2. A mutation in an intron leads to exon skipping

To characterize the molecular defect at the genomic DNA level, we cloned a 3 kb PCR product obtained from a control and containing the patient's deleted mRNA sequence. Sequence analysis of the clone provided information on the intron–exon organization of this part of the *btk* gene. The three introns in this region correspond to nucleotides 1698–1699, 1763–1764 and 1882–1883 (Fig. 2a). The sequence which is deleted from the patient's mRNA is a verbatim copy of an entire exon (referred to here as exon B), suggesting that the genomic defect could lead to skipping of this exon. Comparison of the nucleotide sequences of the corresponding genomic segments in the patient, his mother and the control revealed that exon B is indeed intact in the pa-

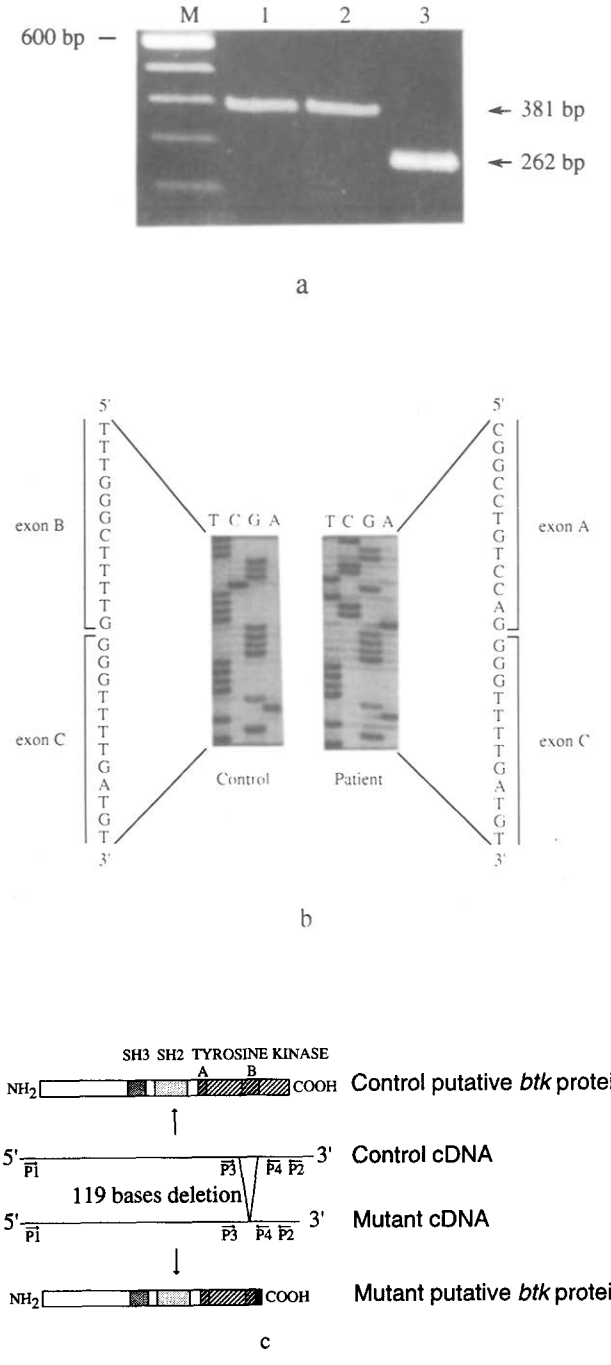

Fig. 1. Identification of a 119 bp deletion in *btk* mRNA. (a) RT-PCR amplification products from a control (lane 1), the patient's mother (lane 2) and the proband (lane 3), using primers P3 and P4. The sizes of the PCR products (bp) are indicated on the right. A 100 bp ladder (BRL), used as a size marker, is in lane M. (b) Nucleotide sequence analysis of the RT-PCR products, using primers P3 and P4, from the patient and a healthy control. The sequence was obtained with primer P4. (c) Diagram of the *btk* cDNA and the putative protein from control and patient. Primers P1, P2, P3 and P4 are indicated. The encoded putative proteins are represented at the top (control) and at the bottom (patient). SH3 and SH2 domains are shown in shaded boxes; tyrosine kinase domain is shown in hatched box, ATP-binding site and substrate-specific domain are denoted by A and B, respectively. The patient's cDNA 119 bases deletion leads to a truncated protein missing the last 61 residues of the carboxyterminal end of the protein. The 14 amino acids changed by the frameshift are shown in black box.

tient's DNA, but that the splice donor site located on the 3′ side contains a G-to-A transition at position + 5 (1882 + 5G→A) (Fig. 2b). The mutation occurred de novo, being absent from the mother's DNA.

3.3. *In vitro analysis of the mRNA processing defect*

To determine whether the 1882 + 5G→A transition in the donor splice site of exon B is sufficient to explain skipping of the exon immediately upstream, normal and variant RNA splicing were analysed in an in vitro system. A 1.4 kb genomic DNA region was amplified from the patient and a normal male with primers P5-B and P6-B, to generate DNA segments covering a region encompassed by the 3′ end of the upstream exon (A) and the 5′ part of the downstream exon (C) (Fig. 3a). These products were then cloned in the splicing vector pSPL1, a plasmid designed to trap coding sequences contained in complex genomic DNA. RNA extracted from COS-7 cells transfected with the pSPL1 recombinants were reverse-transcribed and amplified with PCR primers SD3 and SA4. Two products of different sizes were obtained: a product of 660 bp from the normal pSPL1 recombinant, and a smaller product (541 bp) from the mutant construct (Fig. 3b); the length difference corresponded to that of exon B, absent from the patient's mature RNA, as confirmed by sequence analyses (data not shown).

As another mutation in the intron sequences of the patient's DNA could cause skipping of exon B, the mutated sequence was reverted in the mutant clone by changing the A at position + 5 into a G. This reversion to the wild type restored correct processing of the mRNA (Fig. 3b), demonstrating that the 1882 + 5G→A transition is sufficient to induce skipping of exon B. Furthermore, this mutation perturbed splicing of most of the mutant mRNA sequences since the processed transcripts appeared homogeneous.

4. Discussion

The aim of this study was to understand the molecular mechanism underlying the phenotype observed in a patient with both typical X-linked agammaglobulinemia (XLA) and isolated growth hormone deficiency (IGHD), conditions coexisting in rare families [19–21]. In most cases, these two disorders are not associated. IGHD segregates in an autosomal recessive (IGHD I) or dominant (IGHD II) fashion due to various alterations in GH gene [32] and, in some cases, as an X-linked disease (IGHD III). Genetic linkage studies have previously established that the locus involved in this disease association maps to Xq22, like the XLA locus [22]. Since the patient reported here is a sporadic case, we were unable to demonstrate that the two diseases were inherited together. It is unlikely that the growth hormone deficiency in our patient results from an acquired alteration of the pituitary

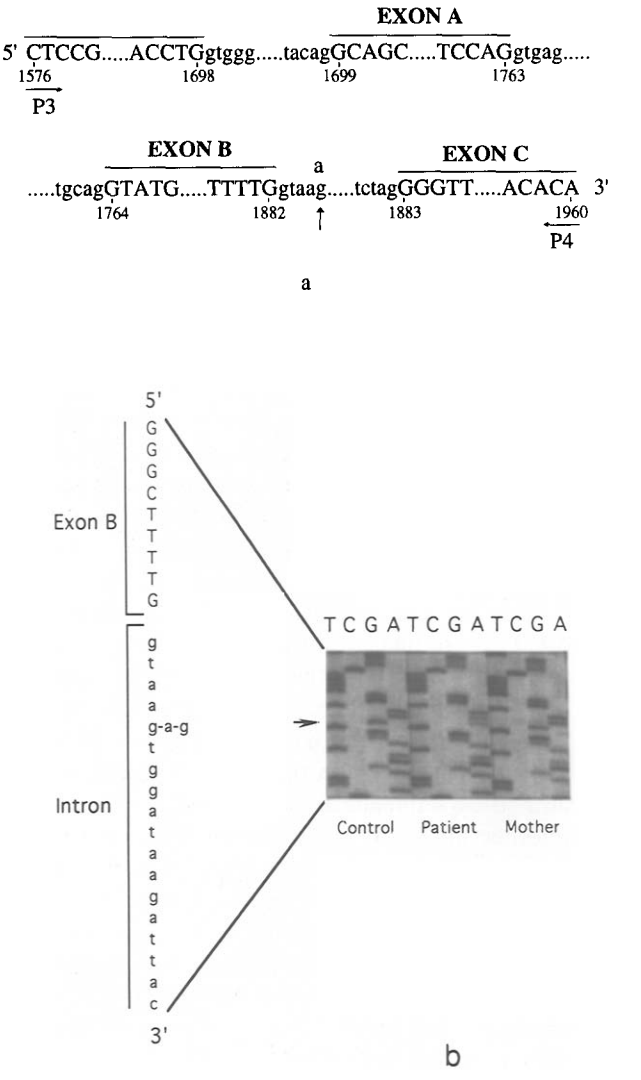

Fig. 2. Nucleotide sequences of the *btk* gene region corresponding to the deleted mRNA sequence. (a) Intron–exon organization. Sequence analysis of a 3 kb PCR product obtained from control genomic DNA, using primers P3 and P4. Intron and exon sequences are denoted by lowercase and uppercase letters, respectively. Nucleotides in exons are numbered according to Vetrie et al. [14]. The G-to-A transition at position + 5 of the splice donor site of exon B in the patient's genomic DNA is indicated by an arrow. (b) Nucleotide sequences around the splice donor site of exon B. The arrow indicates the G-to-A substitution at position + 5 of the splice donor site in the patient, as compared to the patient's mother and a healthy control.

gland since other pituitary functions were found normal. In addition, the two disorders appeared de novo: they are not present in the other members of his large family. The characterization of the *btk* gene, a gene which was recently shown to be responsible for several XLA cases [14,15,17,18] prompted us to seek a possible common developmental or biochemical defect at the *btk* locus which would explain the coexistence of antibody and growth hormone deficiencies.

A search for potential gene rearrangements or deletions in the genomic DNA of this subject by Southern blot analysis after regular and pulse-field gel electrophoresis yielded an unremarkable restriction pattern (not shown). However, careful analysis of the *btk* gene and transcript revealed the existence of a de novo mutation at position + 5 of a splice donor site (1882 + 5G→A) that alters normal splicing, removing the tyrosine kinase domain exon immediately upstream. As revealed by RT-PCR and direct sequencing, this exon skipping event alters all the *btk* transcripts.

Making use of an in vitro splicing test, we obtained evidence that the substituted nucleotide is critical for correct maturation of the *btk* mRNA. The nucleotide at position + 5 in the splice donor sites is generally a guanine in 84% of introns, and an adenine, cytosine or thymine in the remaining 16% [26]. Several mutations at this position have been shown to abolish normal splicing and to result in similar exon skipping processes [27–30], but given the natural occurrence of an adenine in position + 5 in some of 5′ splice sites, it was necessary to demonstrate that the sequence variation found in the patient's

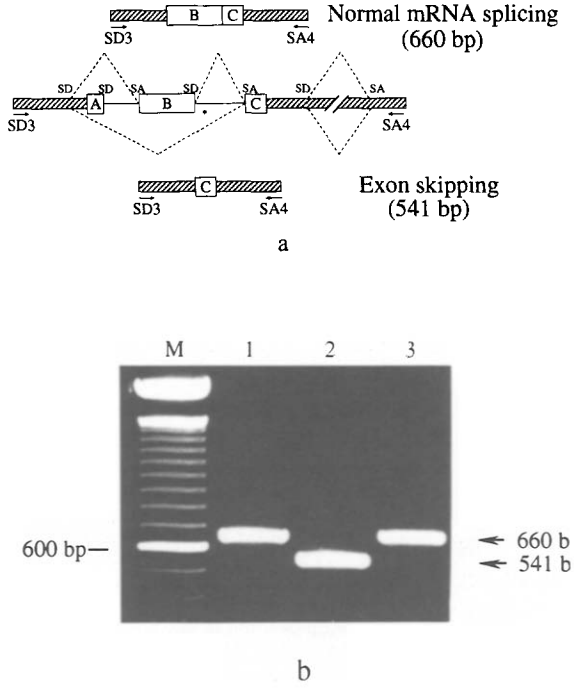

Fig. 3. In vitro analysis of *btk* mRNA splicing in transfected COS-7 cells. (a) Diagram of the genomic DNA fragment amplified with primers P5-B and P6-B and cloned into pSPL1. The G-to-A transition is denoted by an asterisk. The sequences of the vector are shown in hatched boxes. SD and SA indicate the positions of the donor and acceptor splice sites, respectively. Normal and mutant splicing patterns are schematically represented and the sizes of the corresponding spliced products are indicated. (b) RT-PCR amplification products from COS-7 cells RNA prepared after transfection with the various constructs corresponding to the patient (lane 1), a control (lane 2) and the patient after reversion of the mutation to wild-type by site-directed mutagenesis (lane 3). The size of the products is indicated on the right (bp); lane M, size marker.

DNA indeed led to exon skipping. This was done by showing that reversion of the mutant sequence to the wild-type restored correct processing of the *btk* mRNA, thereby establishing that the 1882 + 5G to A transition induces the splice defect. Skipping of exon B results in a truncated *btk* protein in which the last 61 residues of the carboxyterminal end of the protein are deleted. More precisely, this deletion removes the second half of the substrate-specific domain. This domain contains the tyrosine 551, a residue presumed to be a positive regulatory phosphorylation site also found in the other members of this protein tyrosine kinase family and in the members of the *src* and *abl* tyrosine kinase families [14,16,31]. Such truncation is likely to abolish protein function, thereby causing the XLA phenotype.

Various point mutations, insertions and deletions have previously been identified in all domains of the *btk* protein [14,17,18]. Interestingly, in addition to this molecular heterogeneity, the XLA phenotype is variable. Indeed, besides severe agammaglobulinemia cases, milder phenotypes are observed. It is possible that some mutations, which do not completely abolished *btk* protein function could lead to a milder disease, as suggested by Bradley et al. [18]. The mutation described here results in the loss of the substrate-specific and C-terminal domains of the protein, the N-terminal, SH3, SH2 and ATP-binding site domains remaining intact. Two mutations resulting in the loss of the 23 C-terminal residues of the protein have been described in patients with severe agammaglobulinemia [17,18]. However, as compared to the mutation reported in this work, these deletions do not alter the substrate-specific region of the tyrosine kinase domain.

The association of XLA and isolated growth hormone deficiency (IGHD) in this patient is puzzling. Several mechanisms have been proposed to explain this complex phenotype [22]. Firstly, another gene located in the vicinity of the *btk* gene and controlling growth hormone secretion could be missing, but deletion of the *btk* gene along with another contiguous gene was ruled out. In addition, it is highly unlikely that two independent point mutations occurred de novo, one altering the *btk* gene and the other a gene that controls growth, since there is no family history of IGHD, and the mutation in the *btk* gene was not found in the mother. Secondly, among other possibilities is the existence of another gene located in the Xq21.3–q22 region, encoding a factor involved in both the control of pre-B cell maturation and growth hormone expression. However, our results clearly demonstrate that the *btk* gene mutation is responsible for the XLA phenotype in this patient. A third possibility is that some mutant forms of the *btk* gene could give rise to XLA alone, while other mutants could generate both XLA and IGHD. If this were the case, the *btk* gene would probably be expressed in the pituitary gland where GH is produced. To test this hypothesis, 30 cycles of RT-PCR were carried out on mRNA from pituitaries, and the product was sequenced. This led to the detection of a specific *btk* amplification product of expected size and sequence (data not shown). It is thus tempting to speculate that the protein tyrosine kinase encoded by the *btk* gene could play a role in the biosynthesis or secretion of growth hormone, and that some mutant forms of the *btk* protein could impair both the production of growth hormone and the development of B lineage cells. Characterisation of additional *btk* gene mutations in the rare patients inheriting both XLA and IGHD is eagerly awaited.

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