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Isolated growth hormone deficiency type II caused by a point mutation that alters both splice site strength and splicing enhancer function

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Abstract

A heterozygous single base mutation in the human growth hormone (GH) gene (*GH-1*) was identified in a family presenting with isolated GH deficiency type II (IGHD II). Affected individuals have a guanine to adenine transition at the first nucleotide of exon 3 (E3+1 G→A) that results in exon skipping and production of a dominant-negative 17.5-kDa isoform. We show that the mechanistic basis for exon skipping is due to the unique position of this mutation because it weakens the 3' splice site and simultaneously disrupts a splicing enhancer located within the first seven bases of exon 3. A G→T mutation at this same position not only affects splicing but also results in a premature stop codon for those transcripts that include exon 3. Thus, mutations that alter the first nucleotide of exon 3 illustrate the various mechanisms by which changes in sequence can cause disease: splice site selection, splicing enhancer function, messenger RNA decay, missense mutations, and nonsense mutations. For IGHD II, only exon skipping leads to production of the dominant-negative isoform, with increasing skipping correlating with increasing disease severity.

Keywords

GH; IGHD II; mRNA splicing

The human growth hormone (GH) gene, *GH-1*, comprises five exons that are constitutively spliced to produce a full-length, 22-kDa protein that accounts for the majority of circulating GH. Aberrant splicing of wild-type transcripts gives rise to at least five smaller isoforms, the most abundant of which are a 20-kDa isoform and a 17.5-kDa isoform (1). The latter is generated by complete skipping of exon 3 and acts as a dominant-negative isoform (Fig. 1a). The 17.5-kDa isoform exerts a dominant-negative effect by preventing secretion of wild-type GH in both tissue culture cells and transgenic mice (2–5). Patients with inherited mutations that increase the levels of the 17.5-kDa exhibit isolated GH deficiency type II (IGHD II), an autosomal dominant form of GHD. Common characteristics of IGHD II

include short stature due to impaired bone elongation and, in severe cases, anterior pituitary hypoplasia with concomitant disruption of the anterior pituitary hormone axis.

The splice sites surrounding exon 3 in *GH-1* are relatively weak and require the concerted effort of three splicing enhancers to ensure exon inclusion (6). Two of these enhancers reside within exon 3 [exonic splicing enhancers (ESEs) 1 and 2], whereas the third is found in the downstream intron (intrinsic splicing enhancer) (Fig. 1a,c). ESE 1 comprises the first seven bases of exon 3 and is required for both proper recognition of the upstream 3' splice site and suppression of the downstream cryptic splice site (7). Disruption of any of these elements leads to increased production of the smaller GH isoforms including the dominant-negative isoform due to skipping of exon 3 (6).

Here, we examine the sequence requirements to maintain accurate splicing of *GH-1* transcripts by characterizing a heterozygous guanine to adenine transition at the first base of exon 3 (E3+1 G→A). Our analysis shows that this transition mediates its effects in two ways: by disrupting ESE 1 and by weakening the 3' splice site consensus sequence.

Materials and methods

Subjects

We studied members of a Caucasian family presenting with GHD, inherited in an autosomal dominant manner (Fig. 1b). Clinical tests and pedigree analyses confirmed autosomal IGHD II. Sequencing of DNA from family members identified a heterozygous E3+1 G→A transition in the *GH-1* gene in affected individuals (Fig. 1c).

Patient I-I (Fig. 1b) was admitted with short stature and diagnosed with GHD at age 2 years and 5 months. He was born to unrelated parents and on admission was 75.7 cm in height (−4.62 SD score), and his body weight was 9.5 kg. His body mass index (BMI) was 15.7 kg/m². There was a paternal history of GHD (Fig. 1b).

The patient's serum GH level was 0.91 ng/ml (normal: 0.43–2.4 ng/ml) and was not stimulated after provocation tests (GH peaks: 1.12 ng/ml with clonidine and 1.14 ng/ml with arginine; normal response, at least one peak >8 ng/ml). Serum insulin-like growth factor (IGF)-1 was 28.2 ng/ml (normal: 51–303 ng/ml), and serum IGF-1 binding protein (BP) III was 1.2 µg/ml (normal: 0.8–3.9 µg/ml). His thyroid-stimulating hormone (TSH) serum level was 1.18 µIU/ml (normal: 0.36–7.6 µIU/ml). GH, TSH, IGF-1 and IGF-1 BP III analyses were performed using a chemiluminescent immunometric assay with Immulite 2000 (Diagnostic Products, Los Angeles, CA). A cranial magnetic resonance imaging showed a hypoplastic anterior pituitary. Subcutaneous GH replacement therapy (Humatropen; dose 0.23 mg/kg/week) began at 2 years and 6 months and showed a good response to treatment within the first 4 months. By age 4 years and 9 months, the patient was 104 cm (−0.95 SD score) in height, weighed 16.6 kg, and had a BMI of 15.32 kg/m². Following therapy, slight scoliosis of the spine and increased truncal adiposity exhibited before treatment were reduced. At age 5 years and 3 months, the patient is at the 25th percentile for height.

Cell culture and *in vivo* splicing analyses

Blood samples were obtained from the subject, patient I-I, and from an affected cousin (patient I-II; Fig. 1b) after obtaining consent. Genomic DNA was isolated from total blood and the *GH-1* gene sequenced using the following primers: 5'-CCAGCAATGCTCAGGGAAAG-3', 5'-TGTCCCACCGTTGGGCATGGCAGGTAGCC-3' and 5'-CTGGGAAATAAGAGGAGGAGAC-3' (8). Patient lymphoblastoid cell lines (LCLs) were isolated and transformed (9), and total RNA was isolated from 2×10^6 cells using RNeasy (Qiagen, Valencia, CA). Complementary DNA was synthesized (10) and products amplified using ^{32}P -labeled *GH-1*-specific primers to assay *GH-1* splicing patterns (7).

Mutant constructs were generated from wild-type *GH-1* in pXGH5 by reverse polymerase chain reaction (PCR) (11). The *GH-1* open reading frame was maintained in all mutants. Rat somatotroph GH3 cells were transfected with 1 μg of wild-type or mutant constructs and splicing patterns determined (6).

In vitro splicing analysis

Wild-type or mutant ESE 1 sequences were cloned into exon 2 of an enhancer-dependent splicing construct derived from the *Drosophila melanogaster doublesex* gene (*DSX*; a kind gift from Dr B. Graveley) by reverse PCR (11). Splicing reactions and analyses were as previously described (6).

Electroporation of small interfering RNAs

A total of 150,000 LCLs were centrifuged at 2000g for 5 min, resuspended in 75 μl siPORT electroporation buffer (Ambion, Austin, TX) and electroporated with 5 μg of small interfering RNA (siRNA)-17.5 or siRNA-GFP (Dharmacon, Lafayette, CO) in a 1 mm cuvette under the following conditions: single square wave pulse, 325 V, and 13 ms. After electroporation, cells were incubated for 10 min at 37°C before being plated. After 48 h, all cells were reelectroporated under the same conditions. For mock electroporations, LCLs were electroporated in 75 μl siPORT in the absence of siRNAs. Total RNA was harvested after a further 48 h, and *GH-1* splicing patterns were analyzed by reverse transcriptase (RT)-PCR as described above. The sequence for siRNA-17.5 has previously been published (5); the sequence for the control siRNA against green fluorescent protein, siGFP is 5'-GCAAGCUGACCCUGAAGUUCUU-3'.

Results and discussion

A weak 3' splice site increases exon skipping

GH-1 sequences from affected individuals showed a heterozygous single E3+1 G→A transition (Fig. 1a). If translated, messenger RNAs (mRNAs) containing this mutation are predicted to encode a glutamic acid to lysine (E32K) change in the amino acid sequence of GH. However, the majority of mutations that cause IGHD II do so by inducing skipping of exon 3 to produce the dominant-negative 17.5-kDa isoform (12). To determine if the E3+1 G→A mutation actually alters splicing, we transfected GH3 cells with a vector expressing wild-type GH or a construct containing the E3+1 G→A mutation. RT-PCR results show that

when the E3+1G→A mutant was expressed, there was an approximately sixfold increase in skipping (3 transcripts) compared with the wild-type sample (39% and 6%, respectively; Fig. 2a).

One mechanism by which this mutation may affect splicing could be by weakening the 3' splice site of exon 3 from the AG|G consensus to AG|A. For the major class of introns, the AG dinucleotide at the end of introns is absolutely conserved, whereas the G at the first position of exons is found in only ~52.5% of cases (A is 22.5%; C and U are both 12.5%) (13). The initial stages of spliceosome assembly involve the binding of the small subunit of U2 auxiliary factor (U2AF³⁵) to the 3' splice site with a preference for G at the first base of the exon (14–16). Even though all four bases can function as the first base of exons, the preference for guanine by U2AF³⁵ weakens sites that contain other nucleotides. To analyze the effects of base changes in the first nucleotide of exon 3, we also created constructs containing E3+1 G→T and G→C. Compared with the wild-type sequence, all three base changes resulted in increased exon 3 skipping, with the E3+1 G→A mutation causing less skipping (39%) than either of the other mutants, G→T or G→C, 78% and 65%, respectively (Fig. 2a). The E3+1 G→T mutation has previously been reported to cause IGHD II (17). This mutation introduces an in-frame premature termination codon (GAA→UAA) and, consistent with nonsense-mediated mRNA decay [NMD; (18)], no wild-type transcripts were detectable when this construct was expressed (Fig. 2a). Comparing the wild-type, A, and C constructs, it is apparent that there is a preference for G at position 1 of exon 3, followed by A and then C. The effect of T cannot be determined under these conditions because transcripts containing this change cannot be detected, consistent with NMD. Despite this, the results are consistent with the percent that each of these bases is found in consensus 3' splice sites (13).

Splicing analysis in patient-derived LCLs

The experiments shown in Fig. 2a were performed in cultured GH3 cells, which mimic a homozygous mutant background. However, *GH-1* transcripts can be detected in patient-derived LCLs, providing a molecular tool to characterize the effects of specific mutations in heterozygous settings (19). We created LCLs from both affected and unaffected individuals and assayed *GH-1* splicing patterns. As shown in Fig. 2b, the overall pattern of RT-PCR products is slightly different from that observed in transfected GH3 cells due to some additional faint bands migrating close to the band corresponding to transcripts encoding the 20-kDa isoform. While this may slightly alter the percentage of each transcript, it is clear that there is a dramatic increase in 3 transcripts in LCLs from IGHD II patients compared with unaffected individuals. The increase in 3 transcripts in the LCLs is relatively modest compared with the data from transfected rat somatotrophs. This may be due to heterozygosity in the LCLs compared with transfected GH3 cells or to differences in transcript levels between the two cell types. When we compared the levels of 3 transcripts in three individuals with IGHD II, the levels of the dominant- negative 17.5-kDa isoform correlated with disease severity. For the two individuals with heterozygous E3+1 G→A mutations, the levels of 3 transcripts were lower (Fig. 2b) compared with an individual containing a mutation at the 5' splice site of intron 3 (IVS3+1 G→A), in agreement with clinical observations for both types of mutations (20–23). Patients with IGHD II caused by

mutations in either of the first two bases of intron 3 (IVS3+1/+2) have a more severe phenotype with earlier onset than those with mutations within ESE 1 due to increased production of the 17.5-kDa isoform. IVS3+1/+2 corresponds to the conserved GU dinucleotide at the 5' splice site that pairs with U1 small nuclear ribonucleoprotein binding in the initial stages of spliceosome assembly and exon definition. Patients with the E3+1 G→A mutation do not exhibit as extreme a phenotype as patients with mutations at IVS3+1/+2, most likely due to reduced exon 3 skipping and therefore less production of the dominant-negative 17.5-kDa isoform. These phenotypic differences may reflect a threshold and dose dependency of the amount of 17.5-kDa isoform above which pituitary defects are triggered (3, 23).

Disruption of ESE 1 and exon 3 skipping

While the 3' splice site consensus sequence is altered in patients containing the E3+1 G→A mutation, there are many functional 3' splice sites that contain an adenine as the first base of the exon. Another possibility, although not mutually exclusive, is that the E3+1 G→A mutation causes exon 3 skipping by disruption of ESE 1. To test this, we created a series of constructs designed to assay enhancer activity. Single copies of wild-type and mutant ESE 1 were cloned into a construct derived from the *D. melanogaster doublesex (dsx)* gene where splicing is enhancer dependent (Fig. 2c) (24–26). Multimers of small, purine-rich sequences can act as enhancer elements in this setting (24) such that two or more juxtaposed copies of ESE 1 with a single G→A mutation might not alter enhancer activity within this system because the resulting sequence would be extremely purine rich and, importantly, recreate the first six nucleotides of ESE 1 (GAAGAAGGAAGAAG → AAAGAAGAAAGAAG). As a result, we chose to insert only a single enhancer element even though splicing activation was expected to be less than robust. Nevertheless, *in vitro* splicing of RNA transcripts from these constructs revealed splicing activity and therefore enhancer activity upon insertion of a single wild-type ESE 1 (Fig. 2d). In contrast, all three mutant ESE 1 constructs (DSX-ESE 1-A, DSX-ESE 1-T and DSX-ESE 1-C) were unable to rescue splicing. This suggests that altering the first base of ESE 1 destroys its ability to function as an enhancer. Thus, the E3+1 G→A mutation causes aberrant skipping of exon 3 by both altering the 3' splice site and disrupting ESE 1, creating double indemnity.

The E3+1 G→T mutation was identified in a Japanese family with autosomal GHD (17). In that study, the authors postulate that increased skipping of exon 3 occurs for two reasons. First, and consistent with our results, by disrupting the 3' splice site of exon 3. Second, by increased production of 3 transcripts due to nonsense-associated altered splicing (NAS). Initial work that led to the NAS model whereby nonsense codons can promote alternative splicing has not been supported by follow-up experiments (27–29). Further, because all transcripts containing the E3+1 G→T mutation are degraded by NMD, increased detection of 3 transcripts is mostly an indirect result of NMD. Thus, we agree that changes in the first nucleotide alter splice site strength, but our results show that simultaneous mutation of ESE 1 compounds the defect.

RNA interference in LCLs

Transcripts encoding the 17.5-kDa isoform lack exon 3 and thus possess a unique sequence upon joining of exon 2 to exon 4. As a result, the 3 transcripts present an ideal target for knockdown by RNA interference (RNAi) using siRNAs. To determine if RNAi could reduce levels of the 3 transcript in heterozygous patient-derived cells, we electroporated LCLs with siRNA-17.5 directed against the unique exon 2–4 junction. RT-PCR analyses showed specific knockdown of mutant transcripts in three patient lines, whereas an siRNA control (siRNA-GFP) had no effect (Fig. 3). No apparent defects in the rate of cell growth or other obvious phenotypes in the siRNA-treated cell lines were observed, suggesting that off-target effects are minimal (30). This is consistent with recent findings using RNAi to rescue a murine model of IGHD II (5) but extends the analysis to suggest that RNAi could be a viable treatment for IGHDII in humans.

Current treatment for IGHD II involves subcutaneous injections of recombinant GH (rGH) (31). While this enables patients to attain near-normal stature and is quite effective if managed properly, it neither replicates the normal, pulsatile pattern of GH secretion nor does it prevent anterior pituitary hypoplasia (3, 23, 32). In severe cases, there can be pan-pituitary defects with loss of other anterior pituitary hormones over time. Thus, while height can be corrected, follow-up monitoring is necessary to detect and treat additional deficiencies. In addition, there are side effects, albeit rare, associated with rGH therapy (33). RNAi provides an attractive alternative strategy to specifically target transcripts encoding the 17.5-kDa isoform for degradation (5, 6).

Code within a code within a code

Prior to the realization of the multiple effects that mutations can have on RNA processing, it is likely that the effect of the E3+1G→A mutation would be assumed solely due to the E32K missense mutation that is expected to accompany this change. It is not clear whether the E32K change would cause production of non-functional GH, but even if so, there is no evidence that haploinsufficiency can explain IGHD II. Haploinsufficiency has not been shown in heterozygote carriers of individuals harboring *GH-1* deletions, and individuals with a deletion of one of the two *GH-1* alleles do not have GHD. The best explanation is that because the mutation alters the first base of exon 3 and also the first base of ESE 1, the effects of this change, especially related to IGHD II, appear to be due to aberrant splicing rather than to production of a missense form of human GH.

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Fig. 1.

Identification of a heterozygous mutation in human growth hormone (GH) gene, *GH-1*, that causes IGHD II. (a) *GH-1* contains five exons that encode the functional, wild-type hormone. Skipping of exon 3 produces a dominant-negative 17.5- kDa isoform, elevated levels of which cause isolated GH deficiency type II (IGHD II). Patient mutations that disrupt exon 3 splicing are shown as black circles and splicing enhancers are shown as gray boxes. (b) Genetic pedigree showing inheritance of a mutation at the first position of exon 3 (E3+1) in *GH-1*. The genotype for the two alleles at this position is indicated as either homozygous (G/G) or heterozygous (G/A). Lymphoblastoid cell lines were generated from patients I-I and I-II. ■, affected males; ●, affected females; □, unaffected males; ○, unaffected females. (c) *GH-1* sequencing data from an individual containing a heterozygous G→A transition at the first base of exon 3. Empty boxes are exons, lines are introns and exon splicing enhancers (ESEs) are shown in red boxes. Blue asterisk denotes the IVS3+1 G→A mutation used in Fig. 2.

Fig. 2.

E3+1 G→A weakens the 3' splice site of intron 2 and disrupts exonic splicing enhancer (ESE) 1. **(a)** Rat growth hormone (GH)3 cells were transfected with human *GH-1* E3+1 constructs in which the nucleotide at the first position of exon 3 is as indicated above. The wild-type sequence contains a G. RNA was isolated and reverse transcriptase polymerase chain reaction (RT-PCR) was performed using *GH-1*-specific primers in exons 2 and 5. Bands corresponding to the different spliced products are indicated to the left, and the percentages of these products are listed below based on ratios within each lane from three independent experiments. The wild-type transcript encodes the normal, 22-kDa GH protein; the cryptic transcript results from activation of a splice site in exon 3 and encodes a 20-kDa GH isoform; the $\Delta 3$ transcript results from complete skipping of exon 3 and encodes a 17.5-kDa isoform. **(b)** Patient-derived lymphoblastoid cell lines were generated from patients as described in Fig. 1. *GH-1* splicing patterns were determined by isolation of RNA from individual cell lines and RT-PCR as above. IVS3+1 G→A was derived from a patient heterozygous for a mutation in the first base of intron 3. For **(a)** and **(b)**, the percentage of exon 3 skipping is shown in bold with the associated SDs shown below; these data represent three independent experiments. **(c)** Schematic of the *doublesex* (*DSX*) minigene construct showing two exons (boxes) and an intron (line). Wild-type and mutant ESE 1 sequences were cloned into exon 2 (striped box). The first base of ESE 1 (red, bold) was mutated to A, T or C. **(d)** RNAs derived from the constructs above were prepared by *in vitro* transcription followed by splicing in HeLa nuclear extracts. Splicing reactions were subjected to denaturing polyacrylamide electrophoresis with the precursor and products as depicted. Activation of splicing by wild-type ESE 1 is significantly greater than the other four constructs (*p value <0.0001; n = 6 independent experiments).

Fig. 3. Specific targeting of $\Delta 3$ transcripts by RNA interference. Lymphoblastoid cell lines from affected and unaffected individuals as in Figs 1 and 2 were electroporated with small interfering RNA (siRNA)-17.5, siRNA-GFP or mock electroporated. Total RNA was isolated and spliced products amplified using *GH-I*-specific primers. The bands corresponding to different spliced products are shown as in Fig. 2. The percentages of $\Delta 3$ transcripts are shown below with SDs from at least three independent experiments.