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DNA polymorphisms of the *KiSS1* 3′ Untranslated region interfere with the folding of a G-rich sequence into G-quadruplex

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ABSTRACT

KISS1R and its ligand, the kisspeptins, are key hypothalamic factors that regulate GnRH hypothalamic secretion and therefore the pubertal timing. During studies analysing *KiSS1* as a candidate gene in pubertal onset disorders, two SNP and one nucleotide insertion were observed in a 23 nucleotides G-rich sequence located 65 nucleotides downstream of the stop codon. The polymorphisms formed four haplotypes. Biophysical experiments revealed the ability of this G-rich sequence to fold into G-quadruplex structures and demonstrated that the three DNA polymorphisms did not perturb the folding into G-quadruplex but affected G-quadruplex conformation. A functional luciferase reporter-based assay revealed functional differences between 3′UTR haplotypes. These data show that polymorphisms in a G-rich sequence of the 3′UTR of *KISS1*, able to fold into G-quadruplex structures, can modulate gene expression. They highlight the potential role of this G-quadruplex in the regulation of KISS1 expression and in the timing of pubertal onset.

1. Introduction

The normal timing of puberty is genetically determined, although environmental factors are also involved (Towne et al., 2005). Extreme situations of abnormal timing of puberty result from advanced activation of the gonadotropic axis leading to precocious puberty, or congenital gonadotropin deficiency resulting in delayed or no puberty. Both conditions are determined by genetic factors. Precocious puberty resulting from central activation (central precocious puberty; CPP) of the gonadotropic axis is thought to be a complex genetic disease with familial aggregation (de Vries et al., 2004). In contrast, isolated gonadotropin deficiency (isolated hypogonadotropic hypogonadism, IHH) is a Mendelian trait with autosomal, recessive or dominant transmission and variable expressivity (Karges and de Roux, 2005).

Recently, loss-of-function mutations of *KISS1R* have been described as one cause of IHH (de Roux et al., 2003; Seminara et al., 2003). KISS1R is the receptor of kisspeptins, which are highly conserved hypothalamic neuropeptides (Lee et al., 2009). In all species studied to date, central as well as peripheral administration of kisspeptins have been shown to increase plasma levels of LH and FSH (Dhillo et al., 2006; Elizur, 2009; Matsui et al., 2004; Navarro et al., 2005; Shahab et al., 2005). This increase results from kisspeptin-induced GnRH release triggered by KISS1R activation at the cell surface of GnRH neurons (Gottsch et al., 2006; Messager et al., 2005). Several metabolic pathways, neuromediators or hormones, which have previously been described as major regulators of the gonadotropic axis, actually act through the kisspeptin/KISS1R system (Castellano et al., 2006; Smith et al., 2006). It was quickly suggested that the increase in GnRH secretion occurring at the beginning of puberty could be explained by an increase in kisspeptin hypothalamic signaling. Indeed, hypothalamic expression of *KiSS1*, which encodes kisspeptins, as well as kisspeptin activation of KISS1R signaling, increases during the juvenile period (Han et al., 2005; Shahab et al., 2005).

Systematic analysis of patients with gonadotropin deficiency has shown that the frequency of KISS1R loss-of-function mutations is low (Cerrato et al., 2006 and de Roux et al, unpublished results). In addition to GnRH receptor (GnRHR) and KiSS1R, neurokinin

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B (TAC3) and its receptor (TAC3R) have recently been associated to familial IHH (Topaloglu et al., 2009). Few familial cases are not due to either KISS1R, GnRHR, TAC or TACR3 mutations, making *KISS1* a natural candidate gene for the abnormal timing of puberty (Ko et al., 2010; Luan et al., 2007; Silveira et al., 2010). These studies have mainly focused on identifying coding sequence polymorphisms. A P74S substitution leading to a more stable variant of Kisspeptin 54 was found in a Brazilian boy who developed CPP (Silveira et al., 2010), the P81R polymorphism was less frequent in Korean CPP patients (Ko et al., 2010) whereas a P110T substitution was more frequent in chinese CPP patients than in controls (Luan et al., 2007).

During the course to search for *KISS1* mutations in pubertal onset disorders, we found three polymorphisms in a G-rich sequence located within the 3′ untranslated region (3′UTR) of the mRNA. These polymorphisms are organized into four haplotypes in combination with one polymorphism changing the stop codon to a tryptophan, which leads to the synthesis of a 145 amino-acid prokisspeptin instead of 138. 3′UTR sequences are involved in the post-trancriptional regulation of gene expression by modulating mRNA localization, stability and translation (Fasken and Corbett, 2005). Several small nucleotide motifs within the 3′UTR such as CU-rich, GU-rich, U-rich, poly(C), GC-rich, CA-rich sequences were shown to be involved in these process (Fasken and Corbett, 2005). G-rich sequences are highly abundant in the genome, especially in regulatory regions such as promoters or untranslated regions of mRNA. A common feature shared by sequences bearing blocks of consecutive guanines is their capacity to form G-quadruplex structures in-vitro and to bind specific DNA and RNA-binding proteins (Huppert et al., 2008). They are suspected to regulate gene expression through the regulation of the pre-mRNA 3′ end processing when they are located downstream of the transcription end site (Huppert et al., 2008). In contrast, the role of G-quadruplex structures located in the 3′UTR upstream of the transcription end site remains poorly understood.

In this study, we sought to determine whether the G-rich sequence located within the 3′UTR of *KiSS1* may fold into G-quadruplex, and we analyzed the effect of DNA polymorphisms on G-quadruplex folding and on the regulation of *KiSS1* expression.

2. Materials and methods

2.1. Subjects

In order to look for KiSS1 mutations in pubertal onset disorders, several groups of subjects were analyzed. Group 1 was composed of 92 cases of isolated hypogonadotropic hypogonadism (IHH) without anosmia. The IHH subjects were aged between 2 and 53 at the time of diagnosis. Gonadotropic deficiency was suspected at birth in boys born with micropenis or bilateral cryptorchidism, or at over 15 y of age in patients referred for impuberism or infertility. All adult patients had low plasma levels of sexual hormones, normal or low plasma levels of LH and FSH, and normal levels of other pituitary hormones.

Group 2 was composed of 25 CPP cases. This group was defined by the appearance of pubertal signs associated with increased sexual hormone levels before 8 y of age in females and 9 y of age in males. The central origin of the precocious puberty was confirmed by a positive GnRH test.

The controls were subjects aged between 6 and 20 yrs that served as a reference to establish hormonal norms (Leger et al., 2007). The control group (T) was composed of the 50 boys and 50 girls.

When the subjects were older than 7, both parents and children gave written informed consent for hormonal and genetic analyses. The study was reviewed and approved by the Robert Debré Hospital Ethics Committee.

2.2. KiSS1. sequencing

Genomic DNA was isolated from the patient peripheral blood leukocytes. PCR primers were synthesized by Eurogentec (Seraing, Belgium). They were chosen to PCR amplify the 5′UTR, coding sequence and 3′UTR (see Table 1). Genomic DNA (20 ng) was amplified in the buffer provided with the Taq polymerase (Eurogentec) supplemented with 4% (w/v) DMSO, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 0.2 μM of both primers. The DNA was initially denatured for 3 min at 94°C, then 30 cycles of amplification were performed with denaturation at 94°C for 45 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR products were sequenced with the same primers. Nucleotide sequences were analyzed by SeqScape V2.5 (Applied Biosystems, Carlsbad, CA).

2.3. Subcloning of PCR products

Sequence analysis of exon 3 showed complex profiles combining nucleotide deletions and insertions. A PCR fragment with two primers located 80 nucleotides upstream of the acceptor site of exon 3 and 210 nucleotides downstream of the polyadenylation site was therefore subcloned (Stabycloning kit, Eurogentec). Sequences of subclones were then compared.

2.4. FRET analysis

FRET melting experiments were carried out with a double-labelled DNA oligonucleotide synthesized by Eurogentec (Table 2) with a fluorescein/FAM molecule (fluorescent donor) attached to the 5′-end and a tetramethylrhodamine (TAMRA) as the acceptor molecule attached to the 3′-end (De Cian et al., 2005). As the stability of G-quadruplexes highly depends on the nature and the concentration of the cations, FRET was measured in triplicates in a buffer containing 10 mM sodium cacodylate, pH 7.2 and increasing concentration of KCl, NaCl or NH_4Cl from 10 mM to 100 mM. LiCl was added in order to keep the ionic strength constant at 110 mM.

Measurements were performed on a real-time PCR machine (7300 Real Time PCR System, Applied Biosystem) with an oligonucleotide concentration of 0.2 μM in a volume of 20 μL. The FAM tag was excited at 492 nm and the emission fluorescence was collected at 518 nm. The cycling protocol was an initial denaturation at 85°C for 10 minutes in the studied buffer, followed by an overnight incubation at 25°C to leave refolding of G-quadruplex structures. Recording of FRET was performed every minute with a stepwise increase of 1°C/min for 70 cycles from 26°C to 96°C.

2.5. Circular dichroism

Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter. Oligonucleotides (Table 2), at 3 μM strand

Table 1
Sequences of primers used to amplify and to sequence the three exons of the KISS1 gene (NM_002256.3).

Exon	Name	Sequence
1	KiSS1Fw1	5′_GGATGTGATCAGGGAGCTGG_3′
	KiSS1Rev1	5′_CCGTCTTAGAACGGATTCCC_3′
2	KiSS1Fw2	5′_TTGGAGGACTGTCCCTTTTG_3′
	KiSS1Rev2	5′_CCCTCAATGAGTTGCATGTG_3′
3	KiSS1Fw3	5′_CCTCATCTTTCTGTGCCCTC_3′
	KiSS1Rev3	5′_GCAAAAATGAGCCGCAGACC_3′