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SHORT REPORT

Biallelic mutations in the prokineticin-2 gene in two sporadic cases of Kallmann syndrome

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Kallmann syndrome is a developmental disease that combines hypogonadotropic hypogonadism and anosmia. Putative loss-of-function mutations in *PROKR2* or *PROK2*, encoding prokineticin receptor-2 (a G protein-coupled receptor), and one of its ligands, prokineticin-2, respectively, have recently been reported in approximately 10% of Kallmann syndrome affected individuals. Notably, given *PROKR2* mutations were found in the heterozygous, homozygous, or compound heterozygous state in patients, thus raising the question of a possible digenic inheritance of the disease in heterozygous patients. Indeed, one of these patients was also carrying a missense mutation in *KAL1*, the gene responsible for the X chromosome-linked form of Kallmann syndrome. Mutations in *PROK2*, however, have so far been found only in the heterozygous state. Here, we report on the identification of *PROK2* biallelic mutations, that is, a missense mutation, p.R73C, and a frameshift mutation, c.163delA, in two out of 273 patients presenting as sporadic cases. We conclude that *PROK2* mutations in the homozygous state account for a few cases of Kallmann syndrome. Moreover, since the same R73C mutation was previously reported in the heterozygous state, and because *Prok2* knockout mice exhibit an abnormal phenotype only in the homozygous condition, we predict that patients carrying monoallelic mutations in *PROK2* have another disease-causing mutation, presumably in still undiscovered Kallmann syndrome genes.
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Introduction

Kallmann syndrome (KS) combines hypogonadotropic hypogonadism and anosmia or hyposmia, that is, a deficiency of the sense of smell.[1] Anosmia/hyposmia is related to the absence or hypoplasia of the olfactory bulbs and tracts.[2] Hypogonadism is due to deficiency in gonadotropin-releasing hormone,[3] and probably results from a failure of the embryonic migration of gonadotropin-releasing hormone-synthesising neurones to the forebrain.[4] There are familial cases, but most patients present as seemingly sporadic cases. This is a genetically heterogeneous disease. Loss-of-function mutations in *KAL1* (NCBI GeneID: 3730)[5–7] and *FGFR1* (NCBI GeneID: 2260)[8]

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account for the X chromosome-linked form and an autosomal dominant form of the disease, respectively. *KAL1* encodes anosmin-1, a locally restricted glycoprotein of embryonic extracellular matrices,[9] which is likely to be involved in FGF-signalling through FGFR1.[10] Mutations in *KAL1* or *FGFR1* are found in approximately 20% of all KS patients (see).[11]

In 2006, we identified point mutations in either *PROK2* (NCBI GeneID: 60675) or *PROKR2* (NCBI GeneID: 128674), encoding prokineticin-2 and prokineticin receptor-2 (a G protein-coupled receptor), respectively, in a further 10% of KS patients, both familial and sporadic cases.[12] A majority of these individuals had only one mutated allele. However, we found four patients who were carrying different *PROKR2* mutations in the homozygous state (two patients) or compound heterozygous state (two patients). Notably, the two biallelic mutations were also detected in the heterozygous state in other unrelated patients. The large excess of heterozygous patients for *PROKR2* mutations *versus* homozygotes and compound heterozygotes, combined with the observation that only homozygous knockout mice reproduce the KS phenotype,[13] led us to consider the possibility of a digenic or oligogenic mode of transmission of the human disease, especially in heterozygous patients. Although *PROK2* was an obvious candidate, we did not find a mutation of this gene in any of the patients carrying a mutated *PROKR2* allele. One of these patients, however, was also carrying a missense mutation in *KAL1*, indicating probable digenic inheritance in this individual.[12] Notably, a family has been reported since then, in which a KS male was carrying both a deleterious missense mutation in *FGFR1* and an intronic deletion in *NELF* (NCBI GeneID: 26012) that affected splicing.[14]

Biallelic mutations in *PROK2* have not been reported so far, thus leaving open the question of whether a digenic transmission mode should also be expected in KS individuals carrying only one mutated *PROK2* allele. Here, we report on two KS patients presenting as sporadic cases, who carry different *PROK2* mutations in the homozygous state. These are a frameshift mutation, and a missense mutation we had previously reported in the heterozygous state.

Patients

Case 1

Patient A is a male of Turkish origin, born of consanguineous parents (see Figure 1). He was referred at the age of 18 because of the absence of spontaneous puberty. In addition, he suffered from anosmia. On clinical examination, he had a micropenis and infantile testes. The olfactory bulbs could not be visualised by MRI. Endocrinological tests confirmed the presence of hypogonadotropic hypogonadism in the patient.

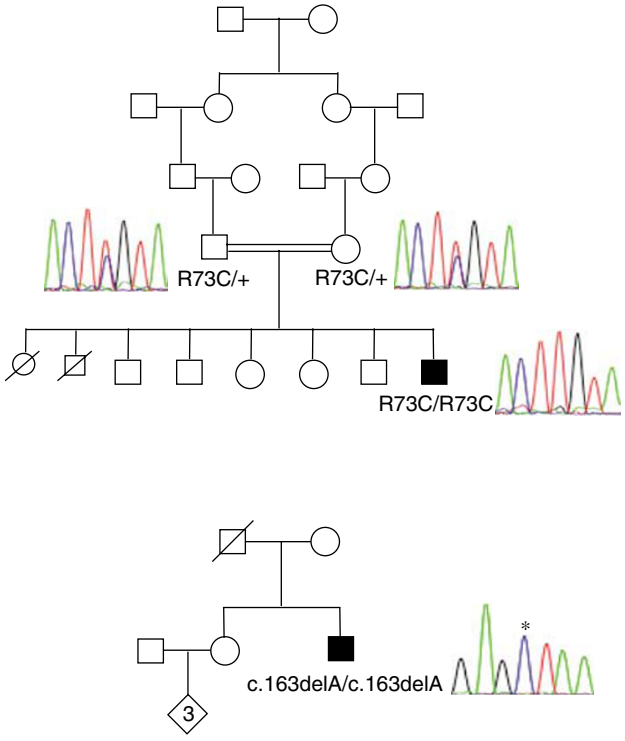

Figure 1 Pedigrees of the two KS patients. Open symbols denote clinically unaffected individuals. Filled symbols denote affected individuals with both hypogonadism and anosmia. The *PROK2* genotypes, if available, are indicated below. The symbol + denotes normal allele. Electrophoretograms of the *PROK2* mutated sequences are shown. The asterisk indicates the position of the nucleotide deleted in patient B.

Case 2

Patient B is a Swiss male, who was diagnosed as KS based on the presence of hypogonadotropic hypogonadism and complete anosmia. The olfactory bulbs could not be visualised by MRI. Virilisation was achieved by testosterone therapy. At the age of 31, however, he had complete azoospermia that persisted after several months of hormonal treatment with gonadotropins.

Methods

Written informed consent was obtained from all the individuals who participated in the study.

Mutation analysis

Genomic DNAs were obtained from blood samples, by using a standard phenol chloroform extraction procedure. Each of the *PROK2* four coding exons and flanking splice sites was PCR-amplified and sequenced using previously reported primers,[12] and standard PCR and sequencing conditions. The mutations were confirmed on a second PCR product. The alleles from 100 control individuals

of Turkish origin were analysed by using a denaturing high performance liquid chromatography standard procedure.

FISH analysis

Peripheral blood lymphocytes from patient B were cultivated for 72 h in medium 199 (Gibco-BRL). Prometaphase chromosomes were prepared by standard procedures, after thymidine synchronisation and BrdU incorporation. They were hybridised with BAC RP11-321A23 (The Wellcome Trust Sanger Institute) that encompasses the *PROK2* gene at chromosome 3p13. DNA from the BAC was Texas red-labelled by nick translation. Chromosomes were visualised by DAPI counterstaining.

Analysis of polymorphic markers

Genotyping was carried out in patient B, with four polymorphic microsatellite markers that span a 8 Mb chromosomal region containing *PROK2*, namely D3S3697, D3S1261, D3S1284, and D3S3581. Standard procedures were used for PCR and automated fluorescent genotyping.

Results and discussion

In patient A, a biallelic missense mutation, c. 217C>T (p.R73C), was found in *PROK2* exon 2. The mutation was also present in the heterozygous state in the patient's seemingly unaffected parents (see Figure 1). This mutation was not found in 200 alleles from ethnically matched control individuals. It produces a cysteine residue, which is expected to disrupt the formation of the protein disulphide bonds. Notably, the same mutation has already been reported in the heterozygous state in a KS sporadic case.[12] No additional mutation was detected in the coding sequences of *PROKR2*, *FGFR1,* and *KAL1* in patient A.

In patient B, a frameshift mutation, c.163delA (p.I55fsX1), creating a stop codon at the same codon position, was detected in *PROK2* exon 2, in a seemingly homozygous state (see Figure 1). The parents' genetic status, however, could not be determined since the father died before this study. To rule out the possibility of a *PROK2* deletion on one chromosome, we carried out a FISH analysis (see Methods) in the patient, which showed the presence of a fluorescent signal at both *PROK2* chromosomal loci (data not shown), thus arguing against a large deletion. We then analysed in the patient four polymorphic markers spanning a 8 Mb interval containing *PROK2* (see Methods), and found that he was carrying only one allelic form of each marker (data not shown). Since patient B is unlikely to have a large deletion of this chromosomal region, he must be homozygous for these polymorphic alleles. This genetic condition could be the consequence of either unrecognised parental consanguinity or unidisomy. We conclude that patient B is most probably homozygous for the c.163delA mutation too. No additional mutation was detected in the coding sequences of *PROKR2*, *FGFR1,* and *KAL1*. While this article was under review, the same *PROK2* mutation was reported in the homozygous state in two brothers affected by KS, and their sister with isolated hypogonadotropic hypogonadism.[15] The premature stop codon is expected to result in mRNA decay or a truncated peptide of 27 amino-acids in its mature form. This peptide was not able to activate the PROKR2 receptor produced by transfected CHO cells.[15]

The presence of *PROK2* biallelic mutations in only two out of 320 patients analysed (273 sporadic cases and 47 familial cases), indicates that this genetic status is rare in KS. In addition, the present findings argue in favour of a digenic inheritance of the disease in patients carrying *PROK2* mutations in the heterozygous state. Indeed, the same R73C mutation in *PROK2* has been identified in the heterozygous[12] and homozygous (this study) states, in two unrelated patients with the same disease phenotype. Moreover, only homozygous knockout mice for *Prok2* have a phenotype that resembles KS, whereas heterozygous mice do not appear to have an abnormal phenotype.[15,16] Finally, the fact that some individuals heterozygous for *PROK2* mutations are clinically unaffected argues against a dominant negative effect of these mutations. Therefore, we suggest that patients heterozygous for *PROK2* mutations carry another disease-causing mutation in a different KS gene. Notably, the same prediction, based on similar findings, was made in patients heterozygous for *PROKR2* mutations, and an additional *KAL1* mutation has been identified in one of them.[12] In the other patients heterozygous for *PROKR2* or *PROK2* mutations, no additional mutation has been found in the KS genes known to date. This suggests that the sought after mutations are located in one or several other genes. Indeed, barely 30% of all KS patients carry a mutation in *KAL1, FGFR1, PROKR2*, or *PROK2*, which indicates that still other genes underlying the disease remain undiscovered. Alternatively, the additional mutations might be located in as yet unexplored noncoding sequences of known KS genes.

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