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Mutational analysis of the human SLC26A8 gene: exclusion as a candidate for male infertility due to primary spermatogenic failure

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SLC26A8 is an anion transporter that is solely expressed in the testes. It interacts with MgcRacGAP that shows strong structural similarity with the *Drosophila* protein RotundRacGAP, which is established to have an essential role for male fertility in the fruit fly. To explore whether the SLC26A8 gene has a role in human male infertility, we performed mutational analysis in the coding region of the SLC26A8 gene in 83 male infertility patients and two groups of controls using single-strand conformational polymorphism and direct sequencing methods. We found six novel coding sequence variations, of which five lead to amino acid substitutions. All variants were found with similar frequencies in both patients and controls, thus suggesting that none of them may be causally associated with infertility. We conclude that the SLC26A8 mutations are not a common cause of male infertility.

Key words: polymorphism/SLC26 gene family/spermatogenesis/TAT1

Introduction

Approximately 10–15% of couples attempting pregnancy meet with failure. The male is estimated to be responsible for approximately 50% of infertile couples. Although multiple causes, such as infections, traumas, malignancies, obstructions etc., have been recognized, the aetiology still remains unknown for up to 40% of cases (de Kretser, 1997). Male infertility has shown to have a major familial component suggesting a role for inherited defects in underlying molecular genetic mechanisms (Meschede *et al.*, 2000). So far, the genetic analysis of male infertility, however, has focused mainly on sex chromosomes, and to date, little is known about autosomal genes and mutations contributing to male infertility via defects in spermatogenesis.

The human SLC26A8 gene is a member of the SLC26 gene family of anion transporters. Its expression is exclusively restricted to male germ line during meiosis, more specifically to spermatocytes and spermatids at different stages of spermiogenesis (Toure *et al.*, 2001; Lohi *et al.*, 2002). It was originally cloned based on its interaction with male germ cell protein MgcRacGAP (Toure *et al.*, 2001). MgcRacGAP demonstrates strong structural similarity with the *Drosophila* protein RotundRacGAP that has proven to be essential for male fertility in the fruit fly (Agnel *et al.*, 1992; Bergeret *et al.*, 2001) suggesting for MgcRacGAP and thus also for SLC26A8 a role in mammalian male fertility. SLC26A8 is located on chromosome 6p21 where the chromosomal breakpoints in infertile males have been reported (Paoloni-Giacobino *et al.*, 2000). The coding region of the human SLC26A8 gene consists of 19 exons that span approximately 80 kb of genomic DNA (Lohi *et al.*, 2002). Interestingly, three members of the SLC26 gene family, namely SLC26A2, -A3 and -A4 if mutated, cause the autosomal recessive diseases diastrophic dysplasia, congenital chloride diarrhoea and goitre associated with congenital deafness (Pendred syndrome), respectively (Hästbacka *et al.*, 1994; Höglund *et al.*, 1996; Everett *et al.*, 1997). This suggests a very essential role for each of the family members in the affected tissues.

Since SLC26A8 is expressed in developing male germ cells only, it is highly possible that the gene is required for the normal development and maturation of this cell lineage. We thus speculated that abnormality and impaired function of the human SLC26A8 gene might contribute to the spermatogenic defects. Our hypothesis was that in order to cause infertility, the mechanism would be autosomal recessive, which is the situation with all diseases caused by the SLC26 family genes (Hästbacka *et al.*, 1994; Höglund *et al.*, 1996; Everett *et al.*, 1997). We thus designed exon-specific intronic primers to amplify each exon separately and have examined the role of the human SLC26A8 gene in spermatogenesis by comprehensive mutational analysis in infertile males with non-obstructive oligozoospermia or azoospermia due to primary spermatogenic failure.

Materials and methods

Patients and controls

DNA samples extracted from peripheral blood samples of 116 infertile Finnish men who had azoospermia or severe oligozoospermia

(sperm concentration $<5 \times 10^6$/ml) were available for the study. The 116 patients were divided into two subgroups based on unknown (study group) versus known aetiology (control patient group) of low sperm counts.

The study group of patients included 83 men with normal male karyotype 46, XY, without Y chromosomal deletions and without any identified aetiology for infertility. The median age of the 83 men in study group was 36 years (range: 25–55 years).

Semen samples had been studied from every patient, and the sperm count not higher than 5×10^6 per ml was the criterion for recruitment. The exact sperm counts were available for this study for 68 men (82%). Of the 68 men, 18 were azoospermic (no sperm in the ejaculate), 26 were extremely oligozoospermic (>0 and $\leq 1 \times 10^6$ spermatozoa/ml) and 20 were severely oligozoospermic (>1.0 and 5×10^6/ml).

The control patient group included 33 men with varying identified aetiology for infertility. Clinical diagnosis in this group included chromosomal abnormalities, such as Y chromosomal deletions and Klinefelter's syndrome ($n = 11$), undescended testis ($n = 7$), obstruction ($n = 4$), mumps orchitis ($n = 2$), operated hernias ($n = 2$), varicocele ($n = 1$), operated hydrocele ($n = 1$), pseudohermaphroditism ($n = 1$), hypogonadotrophic hypogonadism ($n = 1$), trauma ($n = 1$), earlier chemotherapy ($n = 1$) and use of anabolic steroids ($n = 1$). The median age of the 33 group B infertility patients was 38 years (range: 28–49 years). Sperm counts were available for 28 men (85%). Of the 28 men, 14 were azoospermic (no sperm in the ejaculate), 10 were extremely oligozoospermic (>0 and $\leq 1 \times 10^6$/ml) and four were severely oligozoospermic (>1.0 and 5.0×10^6/ml).

Written informed consent was obtained from all participants. In addition, DNAs from 96 healthy Finnish blood donors were used as healthy controls.

PCR

We developed intronic primers for amplifying each exon of the SLC26A8 gene separately. Primers used in the PCR amplification of genomic DNA and the lengths of the PCR products analysed are shown in Table I. Exons 17 and 20 were amplified as two fragments because of their length. All assays work under the following conditions: 94°C for 10 min, 30 cycles of 94°C for 45 s, 58°C (except exon 20B at 62°C) for 45 s and 72°C for 45 s, followed by a final extension at 72°C for 10 min. The PCR amplification was performed in 10 μl volume using 20 ng of genomic DNA template, 0.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, USA), 9 pmol of each primer, 200 uM dNTPs, 2 mM $MgCl_2$, and 1 × buffer provided by the enzyme supplier.

Single-strand conformational polymorphism (SSCP): analysis and sequencing

After PCR amplification, 10 μl of the PCR product was added to 10 μl of bromophenol blue-xylene and formamide containing stop solution. After denaturation at 94°C for 5 min, the samples were placed directly on ice to prevent the reannealing of the single-stranded product, and 2–4 μl of the mix was loaded on a non-denaturing 0.5 × MDE polyacrylamide gel (FMC, Rockland, USA). The samples were electrophoresed at 5 W constant power for 16 h at room temperature followed by visualization by silver staining.

The PCR fragments that demonstrate a mobility shift in SSCP-electrophoresis were recovered with a QIAquick PCR purification Kit (Qiagen, Chatsworth, USA) and sequenced by using an automated sequencer (ABI377A) and dye terminator chemistry.

Screening for sequence variations

For sequence variations V73M, S230N and I639V, the screening and determination of the allele frequencies of the sequence changes in both the patient and the control samples were based on the novel restriction sites that the nucleotide changes generated. V73M generated a novel site for the enzyme *Nsi* I (ATGCAT), S230N for the enzyme *Bsm* I (GAATGCN) and I639V for the enzyme *Hpa* I (GTTAAC). Altered nucleotides are underlined. For sequence variations, I148V and P914S, an artificial restriction site for SNP detection was introduced with a mutated primer. For I148V detection, a novel site for enzyme *Sty* I (CCWWGG) was generated with a reverse primer CATGTCAGACTACGACCATGGTTTCAGTGCTGAACTCACCCA. The restriction enzyme *Sty* I digests the normal allele while the presence of the polymorphism leaves the PCR product intact. To detect the sequence variation for P914S, a novel site for enzyme *Bam* HI (GGATCC) was introduced with a forward primer ATGCATAGGATCAACTGTGTGGGCCCTGAGACTGAGCCTGAGATGGAT. After restriction enzyme digestion, the products were electrophoresed on gel and photographed under UV illumination.

Nomenclature

For the SLC26A8 protein, the designations for the polymorphisms refer to position of the amino acid substitution, where amino acid 1 is the amino terminus of the protein. The cDNA base numbers refer to the cDNA sequence AF331522, where nucleotide 1 is the first A of the first ATG.

Table I. Exon-specific primers

Exon	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
1	CCCACTCTCATCTCCTCAGC	ATTCCACAGATACTGACTGAGTGC	290
2	GGATCTGGATTACTGAAAATGCTC	CAAAGGAGTCAGGTTAGGTTTCAG	310
3	GCATTTTCTTTGGAGAAACTAACC	CAAGGTCTGATACATTGTCTTTGG	239
4	GACAAGCTGCTTTTTCTTAAATTATC	CTGTATGGATAGAATGTCCACCTC	288
5	ACAATCCAGAATTCAACCATACCT	ATCACAGGAGCAGTATAAGGAAGG	357
6	GATTGAAGACATTCTACCCCACTC	GGCATTTGTAACTCTGGGTAAGAG	247
7	GGGACTTCTTTTTGGGTTAATCTAC	GCTTTTACACAATTCAACTCAGCA	326
8	TTAGGTATTCATATGCCCCCATC	GTCAGAGGCTAAGAGAGGGTATCA	191
9	TACCAAGAAAGGATTCCTGCTATC	AAAAAGACTCCTGCTTCATGTTG	283
10	CATATGCCTTCAACTCAAAATCAG	GCATAGCATTAAAACAGCAAGAAG	302
11	AATACGTTCCTGATGGTTTCTCTC	CCTGGTCGTTGTTGACTTAGTTATT	165
12	GCTAACTTTTTGTGATTCCTTGGT	TTTGTTTGTTCTTTCTCCCTCTCT	255
13	GAACTTGTTTTCATGATCATTTTCC	CCTTGCCTTGTTAACCTCCTATTA	192
14	TAATAGGAGGTTAACAAGGCAAGG	CTATCCCTTAGGCAGAGAAATGG	176
15	GAGAAATTTCCCTCAACTTCACAT	AAGGTGAAAGAGTGGGAGAGAGAT	205
16	ATCTCTCTCCCACTCTTTCACCTT	AAATCCCTTAGAGGTTTTGATTTTT	300
17A	GTTTCATGAGAGAGGGACATGAG	CATACTGTTGCCCTTGATTTTTCT	281
17B	TAACCTGATTCACTGCTCACATTT	TAACAGAATTCAATCAGGATCAGG	400
18	ATTAGAAGGCCTTTCCTAGCAAGT	CCAGAACACACACAATGTCAGATA	201
19	TATCTGACATTGTGTGTGTTCTGG	CCTTTCCTGGACATTATTCCTCTA	414
20A	AACTCCTGGTCTAAAGCGATTCT	AGTAACGCTGCTGAGGAAAAGT	440
20B	TGAGACTGAGCCTGAGATGGAG	GGAAGAAGGCTGGCAGGTAGTA	347

Table II. The polymorphisms detected in the human SLC26A8 gene

cDNA change	Codon change	Predicted amino acid change	Genotypes (%) in the study group	Genotypes (%) in the control patient group	Genotypes (%) in control population
G > A	GTG > ATG	V73M	G/G 55.6%, G/A 37.0%, A/A 7.4%	G/G 57.6%, G/A 33.3%, A/A 9.1%	G/G 57.9%, G/A 33.7%, A/A 8.4%
A > G	ATT > GTT	I148V	A/A 66.3%, A/G 32.5%, G/G 1.2%	A/A 63.6%, A/G 36.4%, G/G 0%	A/A 77.0%, A/G 18.8%, G/G 4.2%
G > A	AGT > AAT	S230N	G/G 65.1%, G/A 33.7%, A/A 1.2%	G/G 63.6%, G/A 36.4%, A/A 0%	G/G 76.1%, G/A 20.5%, A/A 3.4%
C > T	GCC > GCT	A257A	Not examined	Not examined	Not examined
A > G	ATT > GTT	I639V	A/A 44.7%, A/G 42.1%, G/G 13.2%	A/A 51.6%, A/G 38.7%, G/G 9.7%	A/A 43.1%, A/G 47.4%, G/G 9.5%
C > T	CCC > TCC	P914S	C/C 97.6%, C/T 2.4%, T/T 0%	C/C 100%, C/T 0%, T/T 0%	C/C 97.9%, C/T 2.1%, T/T 0%

Results

The design of exon-specific intronic primers (Table I) enabled an extensive screening of sequence variations in the male infertility patient material. We screened 83 male infertility patients for nucleotide sequence variations in the entire coding region of the human SLC26A8 gene and the adjacent exon–intron boundaries. The amplified samples were first screened by the SSCP analysis and the fragments suggesting a mobility shift were further analysed by direct sequencing to characterize sequence variations. Finally, the frequencies of the detected sequence variations were confirmed in study group of patients as well as in the samples of a control patient group and in a set of healthy control individuals. This approach resulted in the identification of six polymorphisms in the coding regions of the SLC26A8 gene (Table II).

Polymorphisms in the coding region of the SLC26A8 gene were detected in exons 3, 4, 6, 17 and 20. In addition, the SSCP analysis of the non-coding exon 1 demonstrated mobility shift in both patient and control material, suggesting a polymorphism in the 5′-UTR region, and a substitution of C to T was identified in intron 19 (IVS19-57 C to T). The first sequence variation in coding region of the SLC26A8 gene is a G to A transition at nucleotide 217, which results in the substitution of methionine for valine at position 73 (allele frequencies: G = 0.74, A = 0.26 both in study and control group of patients versus G = 0.75, A = 0.25 in control population). The second sequence change is an A to G transition at nucleotide 442 that causes an isoleucine to valine substitution at amino acid 148 (allele frequencies: A = 0.83, G = 0.17 in study group of patients versus A = 0.82, G = 0.18 in control patient group versus A = 0.86, G = 0.14 in control population). The next two sequence variations when moving towards 3′ were detected in exon 6. At nucleotide 689, a G to A transition leads to a serine to asparagine change at codon 230 (allele frequencies: G = 0.82, A = 0.18 both in study and control patient groups versus G = 0.86, A = 0.14 in control population). In addition, in exon 6 at nucleotide 771, one patient demonstrated a heterozygous C to T change that is silent at the protein level. Allele frequency for this change was not studied. In exon 17, an A to G transition at nucleotide 1915 changes isoleucine at codon 639 to valine (allele frequencies: A = 0.65, G = 0.35 in study group of patients versus A = 0.71, G = 0.29 in control patient group versus A = 0.67, G = 0.33 in control population). The most 3′ sequence variation detected was a C to T change at nucleotide 2734 in exon 20 leading to the substitution of serine for proline at amino acid 914 (allele frequencies: C = 0.99, T = 0.01 both in study group of patients and in control population versus C = 1.00, T = 0.00 in control patient group).

Four of the substituted amino acids do not share homology with other members of the SLC26 family of anion transporters and the only one demonstrating similarity among family members, namely valine at codon 73, is substituted with methionine considered to share similar amino acid characteristics with valine. In addition, valine substituted for isoleucine at codon 148 is present in five family members of nine, thus sharing higher homology than the substituted isoleucine, suggesting the apathogenicity of this amino acid change. Two of the polymorphisms, namely I639V and P914S are located in the areas of SLC26A8-specific sequence segments not found in any other homologous family member, suggested to interfere with the SLC26A8/MgcRapGAP interaction. All variants were found with similar frequencies both in control patient group and in the control population, thus strongly suggesting that none of them may be disease-related. The genotype distributions for these SNPs, determined in the general Finnish population, are shown in Table II.

Discussion

We tested the hypothesis that mutations in the human SLC26A8 gene might be responsible for male infertility in some patients. Mutation analysis revealed six polymorphisms, five of which were predicted to yield amino acid substitutions. All the identified nucleotide changes were observed also in a set of control patients as well as in healthy control individuals and in all groups at relatively high frequencies (one at 1%, but the rest at 14–35%), suggesting that mutations in the human SLC26A8 do not contribute to pathogenesis of male infertility with non-obstructive oligozoospermia or azoospermia. It remains possible that the mutation(s) in an unexamined region of the SLC26A8 gene, such as the promoter region or intron sequences, might be pathogenic leading to disturbed transcription or translation. However, in our previous studies with chloride diarrhoea, we have screened over 108 patients for mutations and found more than 30 different mutations in the coding region of the gene (Mäkelä *et al.*, 2002). Only in six cases, we have not been able to find a disease causing mutation in the coding region of the gene (unpublished data). Thus based on the current knowledge about mutational spectrum with chloride diarrhoea (Mäkelä *et al.*, 2002) as well as with diastrophic dysplasia (Rossi and Superti-Furga, 2001) and Pendred syndrome (Coyle *et al.*, 1998; Van Hauwe *et al.*, 1998; Usami *et al.*, 1999; Park *et al.*, 2003) caused by other SLC26 family genes, we find it rather unlikely that pathogenic mutations in SLC26A8 would occur exclusively in these regulatory regions in a sample of 83 unrelated infertility patients. In addition, in a sample of 83 patients (166 chromosomes) we should expect (power >95%) to see at least one deleterious mutation, if the gene is mutated in approximately 2% of the cases. It is thus a realistic expectation that a number of different mutations would be found in this study, if mutations in SLC26A8 would be reasonably common genetic cause in male infertility. This is further supported by the notion that infertility mutations are rapidly eliminated from the population and thus any infertility gene should show a wide spectrum of new mutations (Reich and Lander, 2001).

The human SLC26A8 gene was in particular attractive as a candidate gene for male infertility, based on its expression exclusively within male germ line during meiosis and its interaction with MgcRacGAP, suggested to have a role in the mammalian male fertility (Arar *et al.*, 1999). However, the oocyte in mouse has shown to activate anion transport activity after fertilization via activating inactive exchangers already pre-existing in the plasma membrane (Phillips and Baltz, 1999). Accordingly, although human SLC26A8 is expressed in the developing male germ cell line, its function, and thus the phenotype caused by its dysfunction, might be redundant or related to a different phenotype than expected and now studied.

The SLC26A8 gene has already been shown to function as an anion transporter mediating at least sulphate, chloride and oxalate transport (Toure *et al.*, 2001; Lohi *et al.*, 2002). It is most likely involved with the transport of bicarbonate, known to have an important role in the activating sperm motility and in the initiation of the acrosome reaction during fertilization. In addition, SLC26A8 is linked to the process of cell division through its interaction with the MgcRacGAP. Further studies are needed to determine its role in the germ cell development *in vivo*.

We conclude that mutations in the SLC26A8 gene are not a common cause of infertility in oligo/azoospermic males. While we have excluded SLC26A8 as a major candidate male infertility gene, we have designed exon-specific intronic primers that enable fast and easy mutation screening of the SLC26A8 gene and used them to identify six novel polymorphisms in the coding region of the gene.

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