No association of the A260G and A386G DAZL single nucleotide polymorphisms with male infertility in a Caucasian population

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BACKGROUND: The human DAZ gene family includes two autosomal genes, BOULE and DAZL, and a Y-chromosomal DAZ gene cluster. All are RNA-binding proteins and assumed to be master regulators of germline gene expression. We have investigated the impact of two DAZL polymorphisms, located at nucleotide positions 260 (SNP 260) and 386 (SNP 386), on the fertility of Caucasian men. These single nucleotide polymorphisms (SNPs) have been described previously to be associated with spermatogenic failure. METHODS: Blood samples were collected and genomic DNA was extracted from 165 normozoospermic men and 202 oligo- or azoospermic patients, of whom 28 displayed an AZFc deletion. The frequencies of A or G allelic variants in SNP 260 and 386 were analysed via TaqMan allelic discrimination assays. In both cases, the A to G transition leads to a threonine to alanine change.

RESULTS: A total of 24.2% of the controls showed a heterozygous nucleotide variant (AG) for the SNP 260 and the remaining 75.8% were homozygous for A. In the AZFc-deleted group, this distribution was significantly different, with 39.3% for AG, 57.1% for AA and 3.6% for GG. However, the increased heterozygosity was not correlated with sperm counts and morphology. The patients without deletions displayed a similar allelic pattern to the controls (24.1% AG/75.9% AA). For SNP 386, only the AA nucleotide variant was found in all subjects studied and in no case was the previously described heterozygous AG variant found. CONCLUSION: In a selected Caucasian population, the DAZL SNP 386 is completely absent and SNP 260 is not associated with spermatogenic failure and therefore does not represent a molecular marker for genetic diagnosis of male infertility.

Key words: DAZL/male infertility/SNP

Introduction
Spermatogenesis is a complex process of mitotic and meiotic divisions of germ cells finally resulting in the formation of haploid spermatozoa. A highly coordinated expression of genes is therefore crucial for normal germ cell development. Recently, a family of RNA-binding proteins, the DAZ (deleted in azoospermia) gene family, was identified. It consists of two autosomal genes BOULE and DAZL (DAZ-like) and a Y-chromosomal DAZ gene cluster. An RNA-binding domain, indicative of RNA–protein interactions, is the main characteristic feature of all DAZ family members (Foresta et al., 2001; Xu et al., 2001, 2003). These genes are expressed exclusively in prenatal and/or postnatal germ cells and are assumed to be master regulators of germline gene expression (Reijo et al., 2000; Xu et al., 2001; Lin et al., 2002). Experimental removal of the autosomal Dazl gene in knockout mice results in infertility caused by a major reduction of testicular stem cells and a failure of spermatogonia to differentiate beyond the early meiotic stage (Schrans-Stassen et al., 2001). Heterozygous Dazl-deficient mice, compared to normal mice, display a higher percentage of malformed spermatozoa indicating a gene dosage effect and that Dazl might also be involved in the control of the major morphological changes during spermiogenesis when the elongated spermatids and mature motile spermatozoa are formed (Ruggiu et al., 1997). Owing to their assumed critical role for maintenance of normal spermatogenesis, mutations or polymorphisms in the DAZ gene family members could result in human male infertility. While AZFc deletions, including all four DAZ gene copies of the Y chromosome, lead to subfertility or complete infertility, associations with male infertility have been described recently for the DAZL gene. Teng et al. (2002) screened 160 infertile men for mutations in the DAZL gene and although the authors did not identify a single mutation, two single nucleotide polymorphisms (SNPs) in the DAZL gene sequence at nucleotide position 260 (exon 2; A→G change) and 386 (exon 3; A→G change) were reported. SNP 386 is located in the RNA-binding domain (RBD) of the DAZL gene. These transitions lead to a threonine to
alanine change at amino acid positions 12 and 54 in the transcribed protein (Figure 1). Analysis of the distribution and frequency of these SNPs among fertile and infertile men belonging to the ethnic group of Chinese Han resulted in a strong association ($P = 0.0003$) of the heterozygous genotype for SNP 386 with spermatogenic failure. No such association could be found for SNP 260 causing a T12A amino acid transition. Since the SNP 386 polymorphism is located in the highly conserved RBD, the authors concluded that this variant could interfere with the RNA-binding function of DAZL. This is the first description of an SNP of an autosomal gene which is associated with a susceptibility for severe spermatogenic failure.

In the current study, we investigated the frequency and distribution of the DAZL gene SNPs in the Caucasian population as a possible molecular predictor for male infertility.

**Material and methods**

**Subjects**

The study was performed on 165 normozoospermic men recruited from clinical studies and 202 oligo- and azoospermic men who were patients attending the Institute of Reproductive Medicine (IRM), Münster. The clinical studies were approved by the ethics committee of the University and the State Medical Board (Münster, Germany; no. 4INie1). All probands and patients gave informed written consent to participate in the study and underwent a complete clinical and physical examination.

The cohort of 165 normozoospermic men is represented by volunteers in contraceptive studies ($n = 69$) or men attending the IRM, Münster ($n = 96$) whose partners were undergoing an IVF treatment due to a female infertility. Men were considered as healthy, normal men by physical examination, medical history, clinical haematology and chemistry, with normal endocrinological profiles [LH, FSH, testosterone, prolactin, estradiol, inhibin B and sex hormone-binding globulin (SHBG)], and provided two semen samples with normal sperm counts, sperm morphology and motility according to the WHO criteria (World Health Organisaton, 1999; Cooper et al., 1992).

The 202 infertile men were patients of the IRM suffering from idiopathic infertility. Exclusion criteria were hypogonadotrophic hypogonadism, chromosomal abnormalities, obstructive azoospermia and infections.

**Blood and semen examination**

Blood samples were taken from all 367 men and serum levels of LH, FSH and inhibin B were analysed by immunofluorometric assays (Autodelfia; Wallac, Inc., Freiburg, Germany). Serum testosterone was measured by radioimmunoassay (Diagnostic Systems Laboratories, Inc., Sinsheim, Germany).

Semen analysis was performed according to the WHO criteria (World Health Organization, 1999). After liquefaction, semen volume, sperm concentration, morphology and motility were estimated.

**PCR assays**

Genomic DNA was extracted from peripheral leukocytes using the Flexi Gene DNA kit (Qiagen, Hilden, Germany). Genetic diagnosis of an AZFc deletion was performed according to the guidelines of the European Academy of Andrology (Simoni et al., 1999). To detect deletions within the different AZF regions of the Y chromosome, the following sequence-tagged site (STS) primers were used in multiplex PCR: sY14 (SRY), sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), and sY254 and sY255 (AZFc). FAM/ VIC were used as an internal control (Simoni et al., 1999). The extension of an AZFc deletion was determined further by using an additional set of STSs (Luetjens et al., 2002).

**Determination of the DAZL SNPs 260 and 386**

For detection of the polymorphisms at nt 260 and 386 we used a method based on the TaqMan technology suitable for allelic discrimination (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Darmstadt, Germany) (Simoni et al., 2002). This allelic discrimination assay is based on a conventional PCR in which two fluorescent probes (short nucleotide sequences) directed to the polymorphism locus studied are additionally included. The probes were labelled with FAM (6-carboxy-fluorescein) or VIC fluorescent dyes (Applied Biosystems) and designed to match perfectly either one or other allele. Fluorescent signals can only be produced when the probe ideally matches to the DNA genomic sequence which thus indicate the presence of one or the other or both SNPs. The primers and probe sequences used for detection of SNP 260 were: forward, 5'-CTTTATGATAGCTTTCTGTTTACT-3'; reverse, 5'-TGGTAAAATAGCTTTGCTTGGTT-3'; VIC-labelled probe, 5'-ACTCAACCTCTCC-3'; and FAM-labelled probe, 5'-AAA-CTCGAGCATCTC-3'. The amplicon size was 71 bp.

Primers and probes for SNP 386 were: forward, 5'-TGGGATCGT-GAATTTTACTCTTGAATTGTTT-3'; reverse, 5'-CAGTGAAGGCAATCT-3'; and VIC probe, 5'-CATAGATG-
ATGAAACTGAGAT-3'; FAM probe, 5' ATAGATGGATGAA-GCTGAGAT-3'. The amplicon size was 96 bp.

Primers for both SNPs were DAZL specific to exclude amplification of the Y chromosomal DAZ genes, e.g. the sequence of the forward primer for the DAZL SNP 260 differs in 7 nt and the sequence of the reverse primer differs in 6 nt from the DAZ sequence. The PCR conditions were as recommended by the manufacturer. Samples of 25 μl reaction volume containing 20 ng of genomic DNA, 5 pmol of each probe and other standard PCR components were amplified under the following conditions: 10 min 95°C, 40 × 15 s 92°C and 1 min 60°C (annealing + extension).

Owing to the lack of patients or control men displaying an SNP 386 G variant, we designed an oligonucleotide of 96 nt (MWG Biotech AG, Ebersberg, Germany) reflecting the total amplicon being amplified by the TaqMan assay and containing G instead of A at nucleotide position 386: 5'-TGA ATG CTG AAT TTT TAC TCT TGA AGT TCA ATT CTT TTC CAT AGA TGG ATG AAG CTG AGA TTA GAA GCT TCT TGG CTA GAT ATG GTT CAG TG-3'. The allelic discrimination assay worked robustly using this oligonucleotide as a positive control. When minute amounts of the oligonucleotide were diluted with genomic DNA of patients AA homozygous for SNP 386, we could detect an AG heterozygous DAZL 386 genotype, confirming that the assay being used is reliably detecting the G allele. A sample containing water instead of DNA, as a negative control, and samples of diagnosed homozygous (AA/GG) and heterozygous men (AG), as positive controls, were used for each PCR run.

Statistical analysis
Data were analysed for statistical significance by two-way ANOVA on ranks and by χ² test using the software program SigmaStat 2.03. The comparison of the SNP 260 distribution in and between the group of fertile men, the group of infertile men with an AZF deletion and the group of infertile men without an AZF deletion was tested using the two-way ANOVA on ranks test and, as a post hoc test, the Turkey test was applied. The frequencies of the SNPs were tested with the χ² test. The frequencies of the SNPs were evaluated according to the Hardy–Weinberg equilibrium and the P-value is given. For all tests, a P-value < 0.05 was considered statistically significant.

Results
Frequency and distribution of the DAZL SNP 260 among Caucasian fertile and infertile men
Using the TaqMan technology we have established an allelic discrimination assay capable of detecting two SNPs in the DAZL gene, SNP 260 and 386. In a Caucasian population, screening of normozoospermic men (n = 165) and infertile men (n = 202) for the DAZL SNP 260 resulted in identification of 93 men displaying a heterozygous 260 AG genotype. In only one patient a homozygous 260 GG genotype could be detected. The overall frequency of the heterozygous 260 AG genotype was 25.3%.

The SNP 260 distribution for the different patient groups is given in Table 1 and Figure 2A. Among the control group consisting of 165 normozoospermic men, 125 (75.6%) were homozygous AA and 40 (24.4%) were heterozygous AG. Within this group, no man was homozygous for SNP 260 G. In the group of infertile men having no AZFc deletion (n = 174), 132 patients (75.9%) displayed the homozygous AA genotype, while 42 men (24.1%) were heterozygous AG. The allelic frequency of SNP 260 among those patients was not significantly different from the control group. Among the 202 infertile men, 45 had non-obstructive azoospermia, 110 severe oligozoospermia (sperm concentration < 1 × 10⁶/ml), 19 reduced sperm concentration (1–5 × 10⁶/ml) and 28 were oligo- or azoospermic with an AZFc deletion. A subdivision of the infertile subjects into azoospermic, severely oligozoospermic and men with reduced sperm counts and recalculation of the haplotype distribution did not reveal a group significantly different from the fertile controls (Figure 2B).

In the group of infertile men with an AZFc deletion (n = 28) 16 men (57.1%) were homozygous for A, 11 (39.3%) displayed an AG heterozygous genotype and one patient (3.6%) was homozygous for G. The frequency of the SNP 260 AG was significantly increased compared with the control group (P = 0.011) but not when compared with the group of infertile patients without an AZFc deletion (Figure 2A).

Table 1. Hormone values (mean ± SEM) of fertile men, infertile men with an AZFc deletion and infertile men without an AZFc deletion grouped according to their DAZL SNP 260 genotype

<table>
<thead>
<tr>
<th>Subject group</th>
<th>DAZL SNP 260</th>
<th>n</th>
<th>%</th>
<th>LH (U/l)</th>
<th>FSH (U/l)</th>
<th>Testosterone (nmol/l)</th>
<th>Inhibin B (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normozoospermic men</td>
<td>AA</td>
<td>125</td>
<td>75.8</td>
<td>3.6 ± 0.1</td>
<td>3.2 ± 0.1</td>
<td>18.9 ± 0.5</td>
<td>180.0 ± 15.2</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>40</td>
<td>24.2</td>
<td>3.7 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>24.9 ± 5.5</td>
<td>206.4 ± 19.0</td>
</tr>
<tr>
<td>Infertile men without</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZF deletion</td>
<td>AA</td>
<td>132</td>
<td>75.9</td>
<td>5.5 ± 0.3</td>
<td>12.4 ± 0.8</td>
<td>17.8 ± 1.8</td>
<td>89.5 ± 11.7</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>42</td>
<td>24.1</td>
<td>5.6 ± 0.6</td>
<td>13.4 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infertile men with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZFc deletion</td>
<td>AA</td>
<td>16</td>
<td>57.1</td>
<td>6.5 ± 0.9</td>
<td>15.9 ± 2.2</td>
<td>24.8 ± 9.6</td>
<td>93.5 ± 27.4</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>1</td>
<td>39.3</td>
<td>5.8 ± 1.1</td>
<td>14.4 ± 2.9</td>
<td>18.5 ± 1.6</td>
<td>87.5 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>1</td>
<td>3.6</td>
<td>4.8</td>
<td>31.0</td>
<td>12.7</td>
<td></td>
</tr>
</tbody>
</table>

LH, FSH and inhibin B levels of infertile men with and without an AZFc deletion (B) differ significantly (P < 0.02) from those of the fertile men group (A).
The identical subject groups as described for the SNP 260 were also screened for the polymorphism SNP 386. However, all men investigated were homozygous AA. We were not able to identify a single patient who was either heterozygous or homozygous for G at nucleotide position 386 of the DAZL gene, a genotype previously described by Teng et al. (2002). To prove that the TaqMan assay for this polymorphism was able to detect the presence of a G variant, we designed a positive control based on an oligonucleotide with a sequence identical to the genomic fragment to be amplified for SNP 386 and bearing at position 386 the nucleotide G. Using this positive control, the TaqMan assay reliably detected the presence of a G at position 386. Thus, methodological problems can be excluded as an explanation for the failure to detect a G haplotype, strongly indicating that the 386 G haplotype was completely lacking in the selected groups.

**DAZL SNP 386**

The finding of abnormal sperm morphology in Dazl-deficient mice prompted us to evaluate whether there was an association of the SNP 260 with sperm parameters such as sperm morphology in fertile and infertile men. However, we did not observe any association between the two SNP 260 genotypes and the percentage of normally formed spermatozoa in the patient collective or in normozoospermic men.

**Discussion**

Male infertility affects ~10% of couples with a desire to have children (Nieschlag and Behre, 2000). Environmental factors or infections contribute to infertility to some extent, but genetic factors also play a pivotal role in causing male infertility. In recent years, several genetic modifications have

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**Figure 2.** (A) Frequency and distribution of the SNP 260 of the DAZL gene among fertile men \((n=165)\) and infertile men with \((n=28)\) or without \((n=174)\) an AZFc deletion. (B) The group of infertile men without AZFc deletion was subdivided further into azoospermic (no sperm), severely oligozoospermic \((<1 \times 10^6 \text{ sperm/ml})\) and oligozoospermic \((1-5 \times 10^6 \text{ sperm/ml})\) men. The frequency of the SNP 260AG in the group of infertile men with an AZFc deletion differed significantly from the control group \((P = 0.011)\).
been identified. The most frequent causes are chromosomal abnormalities or microdeletions of the Y chromosome, while point mutations of essential genes for spermatogenesis seem to be rare. Mutations that severely change the biochemical characteristics of synthesized proteins are not compatible with reproduction and seem not to contribute significantly to male infertility. A novel concept is evolving that SNPs, potentially modifying gene function, might be tolerated in reproduction when their effects are subtle and the frequency among the population is high. Indeed, a number of such SNPs have been reported recently, and for some of them an association with reproductive functions, such as sperm production or different hormone sensitivities, has been shown (Simoni et al., 2002; von Eckardstein et al., 2002).

In agreement with Teng et al. (2002), we failed to demonstrate any association of SNP 260 with spermatogenic impairment. Even when the group of infertile patients was divided into groups with different sperm counts, no association could be found. We were unable to detect the previously described SNP at nucleotide position 386 in the Caucasian group studied. The capability of the TaqMan 386 assay to detect a G variant was confirmed by using a control oligonucleotide bearing a G in its sequence at the position corresponding to nt 386 of the DAZL cDNA. Even when the positive control was mixed with genomic DNA from patients, the assay gave reliable genotype determination. In our opinion, the most probable explanation for the lack of SNP 386G in our study is related to ethnic differences within the collectives. While Teng et al. (2002) investigated men all belonging to the group of Chinese Han, we selected subjects within the Caucasian population.

Comparison of the Y-chromosomal DAZ genes and DAZL in the SNP’s sequence region revealed that all four DAZ gene copies had an A at positions 260 and 386 (Figure 1). No sequence variation within this region of the DAZ genes has been reported yet. In other primates, e.g. in the cynomolgus monkey (Macaca fascicularis), the homozygous A genotype is present in the DAZL gene (Carani et al., 1997; unpublished results). This could indicate that the wild-type DAZL gene is represented by a 260A and 386A genotype. However, no large-scale study has been performed in monkeys yet to exclude that the 260G genotype is completely lacking. The frequency of AG for SNP 260 was significantly elevated (39.3%) in patients harbouring an AZFc deletion compared with the control group (24.2%) (Figure 2A). Moreover, the only patient homozygous for G was identified in the AZFc deletion group. Because of the limited numbers of patients investigated (n = 28), we do not know whether a heterozygous (AG) or homozygous 260G genotype could account for a predisposition to an AZFc deletion.

In this context, it is noteworthy that the transmission of the DAZL SNPs does not follow Mendelian inheritance laws. The P-value of the Hardy–Weinberg equilibrium was <0.00001, demonstrating that none of the genotypes was in accordance with the Mendelian inheritance frequencies. It seems that some selective pressure eliminates the homozygous DAZL 260G variant. Since such a distribution pattern cannot be derived from normal fertilization by germ cells from heterozygous carriers, one might think of pre-selection of germ cells carrying the SNP 260AG. Based on this hypothesis, spermatozoa with the 260G haplotype should not be that competitive in fertilization compared with 260A spermatozoa. Another possibility is that germ cells, presumably primordial germ cells or spermatogonia, containing the homozygous 260G are eliminated and do not contribute to spermatogenesis and subsequent fertilization. Such a scenario is at least possible considering that DAZL not only exerts its function at the onset of meiosis, but is also involved in the migration of primordial germ cells (PGCs) and quantitative aspects of spermatogenesis (Reijo et al., 2000). It also implies that the 260A haplotype is advantageous for the efficiency or qualitative aspects of germ cell proliferation compared with the 260G haplotype. Such a selection of germ cells has been assumed recently for patients suffering from Apert’s disease, which is caused by mutations in the FGRF2 gene. Similar to our findings for the DAZL gene, sperm do not display the expected 1:1 ratio in patients heterozygous for the reported SNP in the FGRF2 gene, but instead display a shifted distribution pattern towards one variant. This is consistent with a pre-meiotic selection, because elimination of a certain haplotype will change the SNP ratios (Goriely et al., 2003). The hypothesis that such a selection exists could be resolved by analysing single sperm cells from men heterozygous for the investigated SNP, but, due to the lack of semen samples in this study, this could not be proved or disproved.

Hormonal analysis of the patients investigated revealed elevated FSH and decreased inhibin B levels compared with the control group, while LH and testosterone were in the normal range. An elevation of serum FSH in the presence of decreased inhibin B is generally acknowledged as an indication of spermatogenic failure, and it has also been shown in other studies (Luetjens et al., 2002). Neither in the control nor in the infertile group could a significant association of hormone levels with the DAZL genotype be found. Thus, the endocrine regulation of spermatogenesis is not influenced by DAZL SNPs.

In conclusion, we have demonstrated that in a selected Caucasian population of fertile and infertile men, the presence of the DAZL SNP 260 is not associated with male infertility. The frequency of the AG heterozygosity was significantly elevated solely in the group of infertile men with an AZFc deletion compared with the control subjects. The distribution of the DAZL SNP 260 did not follow Mendelian laws, considering the fact that the G homozygous variant was nearly completely lacking. In our collective, we failed to show the presence of the previously described SNP 386, which might be due to different ethnic populations studied. We conclude that the DAZL SNPs are not associated with spermatogenic failure and therefore do not represent a molecular marker for genetic diagnosis of male infertility. These results corroborate recent studies on SNPs in other infertility candidate genes, e.g. CAG repeat number in the androgen receptor and polymorphisms in the FSH receptor gene, for which no association with infertility could be shown (Zitzmann and Nieschlag, 2003). In our opinion, SNPs
per se could not be responsible for male infertility and could only act as a co-risk factor.

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