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# DAZL polymorphisms and susceptibility to spermatogenic failure: an example of remarkable ethnic differences

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## Summary

Polymorphisms in genes involved in spermatogenesis are considered potential risk factors for male infertility. Recently a polymorphism in the deleted in azoospermia-like (DAZL) gene (T54A) was reported as susceptibility factor to oligo/azoospermia in the Chinese population. DAZL is an autosomal homologue of the Y chromosomal DAZ (deleted in azoospermia) gene cluster and both are considered master regulators of spermatogenesis. The aim of the present study was to screen (i) for mutations of the entire coding sequence of the DAZL gene in patients lacking of the DAZ gene cluster, in order to evaluate if DAZL polymorphisms may influence the AZFc deletion phenotype; (ii) for the two previously described (and eventually newly identified) single nucleotide polymorphisms (SNPs) in a large group of infertile and normospermic men of Italian origin. We failed to detect new mutations. We confirmed previous results showing no evidence for a functional role of the T12A mutation. Surprisingly, the T54A polymorphism, which was present in 7.4% of the Chinese patients was absent in our Caucasian population. This remarkable difference represent an example of how ethnic background is important also for polymorphisms involved in spermatogenesis and contributes to better select clinically relevant tests, specifically based on the ethnic origin of the infertile patients.

**Keywords:** DAZL, genetics, male infertility, polymorphism, spermatogenesis

## Introduction

The list of potential and ascertained factors influencing male fertility is steadily increasing mainly because of recent progresses achieved in molecular genetics. Among them Y chromosome microdeletions represent one of the most frequent causes of oligoasthenoteratozoospermia (5–7%) and azoospermia (12–15%) (Krausz *et al.*, 2003). The Y microdeletions associated with infertility occur in specific regions of the long arm of the Y, called azoospermia factor (AZF) regions (Vogt *et al.*, 1996; Repping *et al.*, 2002). A strict

genotype-phenotype correlation is observed only for the deletion of the entire AZFa and AZFb regions which are associated with the complete absence of germ cells in the testis and spermatogenic arrest (in general at the stage of spermatocytes), respectively (Vogt *et al.*, 1996; Krausz *et al.*, 2003). On the contrary, the deletion of the most distal AZFc region is associated with a heterogeneous phenotype in different individuals, ranging from the absence of germ cells in the testis to a severe reduction of sperm number/motility and morphology in the ejaculate (oligoasthenoteratozoospermia) (Krausz *et al.*, 2001a). This phenomenon suggests that although spermatogenesis may start without AZFc genes, their absence is crucial for a quantitatively and qualitatively normal spermatogenesis. This region contains a

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total of eight gene families (BPY2, CDY1, DAZ, TTY3.1, TTY4.1, TTY17.1; CSPG4LY and GOLGA2LY) (Skaletsky *et al.*, 2003). The first multicopy gene identified in the region was the deleted in azoospermia (DAZ) which belongs to a gene family that consists of the two autosomal single copy genes: BOULE and DAZ-like (DAZL) and the Y specific DAZ (Reijo *et al.*, 1995; Cooke *et al.*, 1996; Yen *et al.*, 1996; Yen, 2004). All members of the family are expressed exclusively in the germ cells and encodes proteins containing a highly conserved RNA-type RNA-recognition motif (RRM) and a unique DAZ repeat of 24 amino acid residues (Habermann *et al.*, 1998; Reijo *et al.*, 2000; Shan *et al.*, 1996; Xu *et al.*, 2001; Yen, 2004). Besides structural homologies, the abilities of the human BOULE gene to rescue the spermatogenic defect in *Drosophila* boule mutants (Xu *et al.*, 2003), and human DAZ and DAZL transgenes to partially rescue the mouse *Dazl* null phenotype (Slee *et al.*, 1999; Vogel *et al.*, 2002) indicate that the functions of these genes are also highly conserved between species. Taken together these data, it is plausible that the absence of the DAZ gene, because of AZFc microdeletion, can be partially compensated by a fully efficient DAZL gene or in alternative mutations/polymorphic variants in the DAZL gene may lead to a more severe phenotype in AZFc deleted patients. DAZ was copied to the Y chromosome relatively recently, in the old world primate lineage and is 90% identical to its autosomal ancestor DAZL (Saxena *et al.*, 1996). No mutations for the DAZL and BOULE genes have been reported so far, except two single nucleotide polymorphisms (SNPs) in the DAZL gene sequence in exon 2 and exon 3 (Teng *et al.*, 2002). In the Chinese population a significant association between the presence of the SNP (A to G transition) in exon 3 at nucleotide position 386 and oligozoospermia ( $p = 0.0003$ ) has been found whereas the SNP (A to G transition) at nucleotide position 260 was similarly distributed among infertile (3.52%) and control men (2.59%). The mutation in exon 3 is situated within the highly conserved RNA-recognition motif domain of the DAZL protein and leads to Thr54 → Ala change (T54A). Thr54 is conserved for both DAZL and DAZ and it is likely that this mutation may lead to functional consequences such as reduced RNA binding capacity. This would explain the observed similarity in the phenotypic expression of the T54A mutation and the DAZ gene (AZFc) deletion.

The aim of the present study was to evaluate, through mutational analysis of the entire coding sequence of the DAZL gene, the influence of the two known and eventually of newly identified mutations on the spermatogenic potential of AZFc deleted men. In addition, in order to establish if any of the previously described SNPs may represent a risk factor for spermatogenic failure and thus could be proposed as a new diagnostic test, we analysed a large group of infertile and control normospermic men of Italian origin.

## Materials and methods

### Subjects

The study population consisted of 242 patients of Italian origin seeking complete andrological work-up for couple infertility at the Andrology Unit of the University Hospital Careggi (Florence). All patients underwent comprehensive andrological examination including semen analysis, karyotype and Y chromosome microdeletion screening. At the end of the clinical work-up the patients have been divided into three categories: (i) 'idiopathic' ( $n = 92$ ): patients with no abnormal andrological findings in their medical history and at the medical examination, normal hormone levels and normal genetic tests; (ii) 'co-factor+' ( $n = 61$ ) presence of co-factors with potential negative effect on the spermatogenesis i.e. unilateral varicocele, previous infections of the urogenital tract, heavy smoking, monolateral cryptorchidism; (iii) 'non-idiopathic' ( $n = 89$ ): presence of abnormal andrological findings with clearly established negative effect on spermatogenesis i.e. karyotype abnormalities, obstructive azoospermia, bilateral cryptorchidism, bilateral second to third grade varicocele, iatrogenic, bilateral orchitis, hypogonadotropic hypogonadism.

A selected group of 12 patients bearing complete AZFc deletions has been screened separately.

Patients in each category ('idiopathic', 'co-factor+' and 'non-idiopathic') have been further divided into subgroups on the basis of their sperm count: azoospermic (the complete absence of spermatozoa), cryptozoospermia (<1 million spermatozoa/mL); severe oligozoospermia (1–5 millions spermatozoa/mL), moderate oligozoospermia (5–20 millions spermatozoa/mL), asthenoteratozoospermia (total progressive motility <50% with total normal morphology <30%) pure teratozoospermia.

A total of 229 Italian men with normal sperm parameters have been recruited mainly from the Florence area. Ninety men fathered at least one child or had normal fertilization after IVF for pure tubal factor infertility.

All subjects gave an informed consent for molecular analysis of their blood samples.

### Semen analysis

Semen analysis has been performed according to the World Health Organization (WHO) (1992) guidelines at the Andrology Laboratory of the University Hospital of Careggi (Florence).

### Molecular analysis

**DNA source.** The DNA has been extracted from peripheral lymphocytes in the infertile group and in 129 controls whereas DNA was isolated from frozen semen in 100 controls.

For detection of the polymorphisms at nucleotide position 260 and 386 we performed polymerase chain

reactions (PCRs) in 25 µL containing 200 ng of genomic DNA, 10 mM Tris-HCl (pH 8,3), 50 mM KCl, 0.1% Triton X-100, 200 µM deoxynucleotide triphosphates, 100 pmol of each primer, and 1 U Taq DNA polymerase (Promega Corp., Madison, WI, USA). DNA samples were amplified at following conditions: 5 min 94 °C; 30X 30 sec 94 °C, 30 sec 58 °C, 30 sec 72 °C; 5 min 72 °C. The primer sequences used for detection of SNP260 in exon 2 and SNP386 in exon 3 are reported in the paper by Teng *et al.* (2002).

The mutation in exon 2 leads to a Thr12-Ala change (T12A) of the DAZLA protein. The T12A variant creates a DdeI restriction site (CTNAG). On digestion, gel electrophoresis, in agarose gel, showed 67- and 197-bp fragments for the variant, instead of the 264-bp fragment of wild-type allele. Sequence analysis was performed with automatic sequencer (ABI 310; PE Applied Biosystems, Foster City, CA, USA) for all patients presenting the 'A' variant.

The mutation in exon 3 leads to a Thr54-Ala change (T54A) of the DAZLA protein. Sequence analysis was performed with automatic sequencer (ABI 310; PE Applied Biosystems) in 242 infertile and 100 normal controls.

#### Statistical analysis

Statistical analysis has been performed using the statistical package SPSS for Windows (version 11, SPSS Inc. Chicago, IL, USA). All variables were checked for normal distribution by Kolmogorov-Smirnov one sample test.

For comparisons of mean values between groups of different genotypes Student *t*-test for independent samples, when normal distribution was observed, has been applied. Logarithmic transformation of data has been performed in order to normalize the distribution when the presence of log normal distribution was checked. Finally, in case of non-normalized distribution, the nonparametric test Mann-Whitney *U*-test was applied to achieve the same objective.

Allele frequencies were calculated by the allele counting method and the Hardy-Weinberg equilibrium was confirmed  $\chi^2 = 0.6291$  and  $0.5706$  for patients and controls, respectively). Differences in genotype and allele frequencies between patients and controls were compared by chi-square test and Fisher's exact test was used when appropriate.

A *p*-value <0.05 was considered statistically significant in each situation. The null hypothesis of means (or median) equality has been refused.

## Results

#### Mutation screening by direct sequencing of the DAZL gene in patients affected by AZFc deletions

In order to evaluate an eventual modulating effect of DAZL variants or mutations on the AZFc phenotype, a mutational analysis of the entire coding sequence of the DAZL gene has been performed in 12 patients affected by

**Table 1.** DAZL genotypes in 12 patients bearing AZFc deletions. Sperm analysis has been performed according to WHO guidelines (WHO, 1992). Total number (millions spermatozoa/mL); motility (% of type a + b); morphology (% of normal forms)

Code	Y chromosome deletion	DAZLA SNP 260	Semen parameters		
			Total number	Motility (%)	Morphology (%)
A1	AZFc	AA	0		
A21	AZFc	AA	0		
A50	AZFc	AA	0		
A57	AZFc	AA	0.01		
A81	AZFc	AG	0.01		
A116	AZFc	AA	0.01		
A129	AZFc	AA	1.20	4	0
A189	AZFc	AA	0		
A208	AZFc	AA	0.01		
A282	AZFc	AA	1.4	5	0
A302	AZFc	AA	1.8	6	4
A336	AZFc	AA	0		

complete AZFc deletions. No new mutations or SNPs have been detected. In one patient with cryptozoospermia the previously described SNP260 (A to G transition) was found in heterozygosis. The genotype and semen parameters of these patients are reported in Table 1.

#### Search for polymorphisms (T12A and T54A) in the Italian population

*Frequency and distribution of the T12A (SNP260) allele of the DAZL gene in controls vs. patients.* The most frequently observed allele in both controls and patients was the common 'T' allele (Teng *et al.*, 2002). The variant T12A was found both in homozygosis and heterozygosis.

The frequency of the SNP260 genotypes (AA; AG; GG) in the control and the entire patient population (including idiopathic, cofactor+ and non-idiopathic patients) was comparable. A further subdivision of the patient population on the basis of the aetiology and semen parameters was unable to identify significant differences for any of the subgroups vs. controls (Table 2).

*Effect of SNP260 on sperm parameters.* In order to assess if the presence of the 'G' substitution (genotypes AG and GG) is able to influence spermatogenesis or sperm function, we compared the mean values of the three principal sperm parameters (concentration, morphology and motility) among men with and without the 'G' substitution. The mean values of the three parameters were not significantly different between the two groups, in both normospermic and infertile men (Fig. 1a,b).

**Table 2.** DAZL genotypes in the whole study group. Values in parenthesis are expressed as percentages

	Group	DAZL genotypes			Total (n)	p-value vs. controls
		AA	AG	GG		
Classification criteria	Controls	176 (76.8)	48 (20.9)	5 (2.2)	229	
	All patients	173 (71.5)	65 (26.8)	4 (1.65)	242	0.332
Aetiology	Idiopathic	64 (69.6)	26 (28.3)	2 (2.2)	92	0.387
	Cofactor+	44 (72.1)	17 (27.9)	0 (0)	61	0.332
	Idiopathic and cofactor+	108 (70.6)	43 (28.1)	2 (1.3)	153	0.260
	Non-idiopathic	65 (73.0)	22 (24.7)	2 (2.2)	89	0.729
Sperm count* (millions/mL)	0–1	40 (76.9)	11 (21.1)	1 (1.9)	52	0.993
	<5	45 (69.2)	19 (29.2)	1 (1.5)	65	0.368
	>5	23 (63.9)	13 (36.1)	0 (0)	36	0.114

\*Non-idiopathic infertile patients have been excluded.

*Frequency and distribution of the T54A (SNP386) allele of the DAZL gene in controls vs. patients.* Direct sequencing of exon 3 (Fig. 2) has failed to detect the A to G transition in nucleotide position 386 both in the control and patient group. This finding indicates a sharp contrast with the relatively high frequency of the T54A allele in the Chinese infertile population (7.39%).

## Discussion

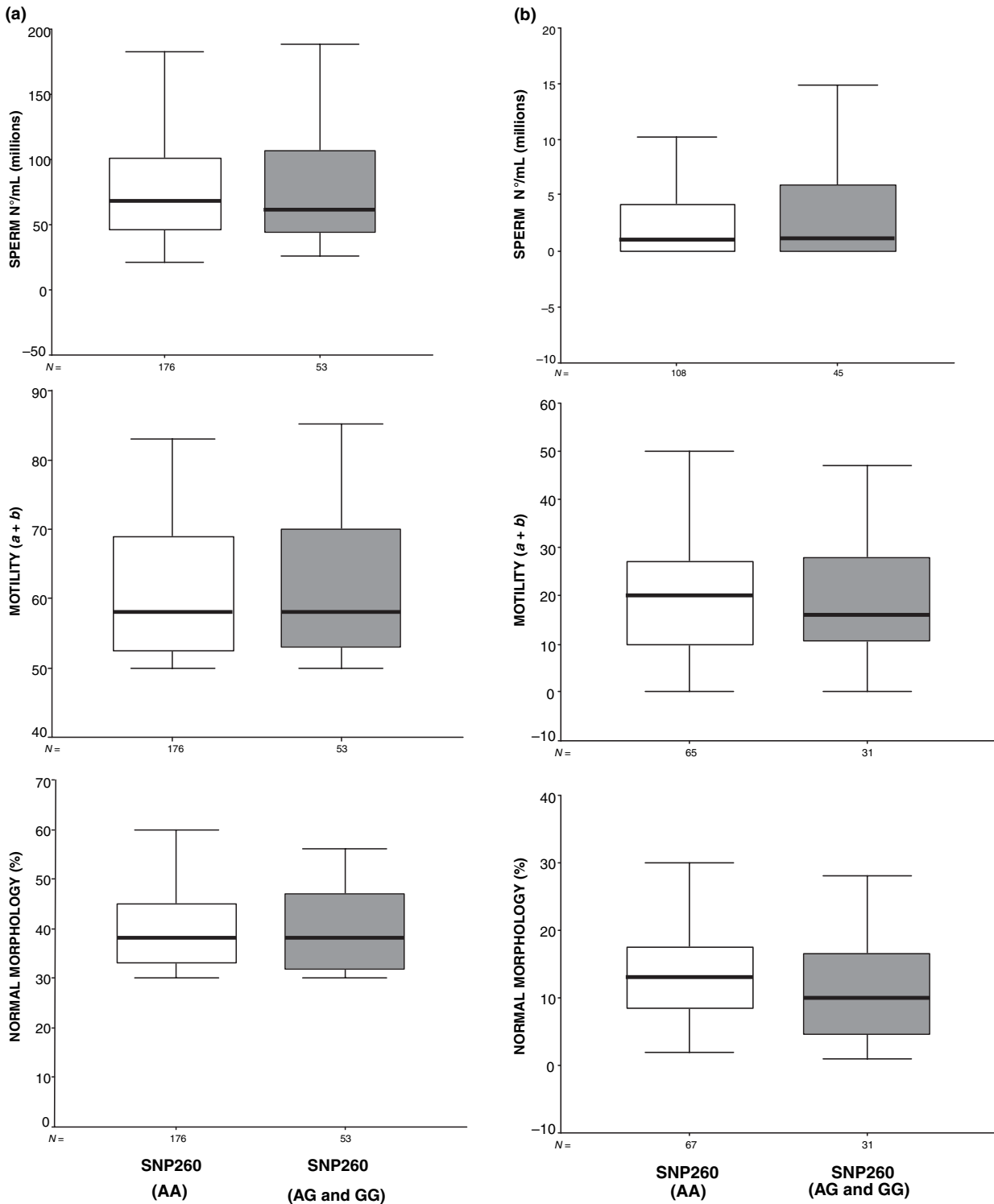
Male infertility is a multifactorial pathological condition mainly (approximately 70%) because of factors acting directly in the testes (Forti & Krausz, 1998). Although some of them induce a homogeneous phenotype (complete AZFa and AZFb deletions of the Y chromosome), the majority of pathogenic factors (Klinefelter sdr, cryptorchidism, chemo/radiotherapy, varicocele, environmental toxic factors, AZFc deletions) cause different degree of spermatogenic failure in different individuals. This interindividual variability is likely to be related to the genetically determined spermatogenic potential of each individual.

Polymorphisms or genetic variants in genes involved in spermatogenesis are considered potential risk factors which may contribute to the severity of spermatogenic failure. Several polymorphic variants have been described in association with oligo/azoospermia (Fritsche *et al.*, 1998; Dowsing *et al.*, 1999; Ruiz-Pesini *et al.*, 2000; van der Ven *et al.*, 2000; Holyoake *et al.*, 2001; Krausz *et al.*, 2001a,b; St John *et al.*, 2001; Kukuvtis *et al.*, 2002; Rajpert-De Meyts *et al.*, 2002; Tsujimura *et al.*, 2002; Asatiani *et al.*, 2003; Tanaka *et al.*, 2003; Yong *et al.*, 2003), and among them a DAZL polymorphism (T54A) has been reported as a susceptibility factor to oligo/azoospermia in the Chinese population. This mutation represents the first example of a SNP in an autosomal gene influencing human spermatogenesis. Independently from this finding, the presence of the

T54A allele does not worsen the phenotype of AZFc deleted men (Teng *et al.*, 2002). However, the lack of effect in this selected group of patients can be related to the influence of other AZFc homologous genes.

Data on DAZL mutations are limited in the literature as only one population has been screened so far (Teng *et al.*, 2002). Therefore, in order to assess whether the two known mutations/variants may influence the spermatogenic potential of AZFc deleted men, and to explore for the presence of yet unidentified mutations, we performed a mutational analysis of the entire coding region of the gene in 12 patients bearing the AZFc deletion. The screening did not lead to the detection of new mutations, and the only variant found was the G to A transition in exon 2 (SNP260) in one cryptozoospermic patient. Only three of 12 patients had a less severe grade of spermatogenic failure with respect to the heterozygous patient, which suggests little or no functional effect of this variant on spermatogenesis.

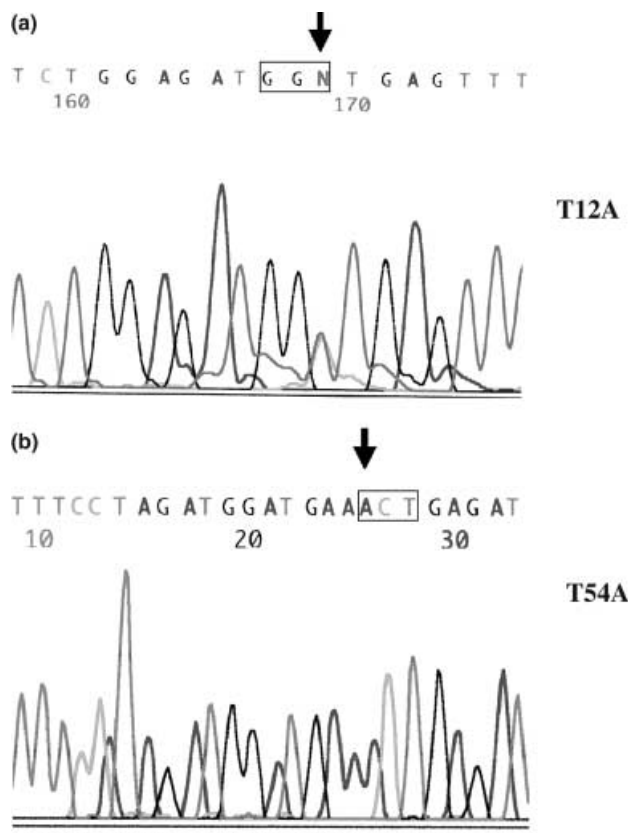
The screening for the two previously described SNPs in a large group of patients and normospermic men gave a surprising outcome. The previously described susceptibility polymorphism, T54A reported to be present at a relatively high frequency in the infertile group (7.39%) and significantly different from that observed in the control group (0.86%), was completely absent in a Caucasian population such as the Italian population. On the contrary, in accordance to the Chinese study, the frequency of SNP260 was not different between infertile and control patients. Given that the group of infertile men was constituted by a heterogeneous population both for aetiological factors and sperm counts, we further subdivided the study population and compared them with the control population. Nevertheless, no specific 'at risk' subgroup has been identified. This data, together with the absence of significant differences concerning the mean



**Figure 1.** Comparison of sperm parameters among men bearing homozygous wild type alleles vs. subjects with the mutated allele (a) in normospermic controls (b) in patients with idiopathic and 'cofactor+' infertility. No significant differences has been observed for any of the three parameters evaluated, both in controls and patients in relation to the SNP genotypes. Sperm analysis has been performed according to WHO guidelines (WHO, 1992). Total number (millions spermatozoa/mL); motility (% of type a + b); morphology (% of normal forms).

values of the three sperm parameters (sperm count, motility and morphology) in both the infertile and in the control groups and the presence of five homozygous

mutants with normozoospermia, suggests that this polymorphism has no evident functional role in spermatogenesis.



**Figure 2.** Direct sequencing of the PCR products (a) exon 2 of DAZL showing an A to G transition in nucleotide position 260 (SNP260) resulting in a threonine (ACC) to alanine (GCC) substitution at codon 12 (T12A); (b) in exon 3 no transition has been observed in our study population at nucleotide position 386 (SNP386). Arrows indicate the previously reported two polymorphic sites.

Our study, based on a large group of infertile and normospermic men clearly indicates that the screening for T54A (and T12A) does not provide a new diagnostic tool in

a Caucasian population. A similar conclusion has been made by another preliminary study dealing with a low number of patients ( $n = 95$ ) and controls ( $n = 65$ ) from Caucasian origin and using a different method for the detection of this polymorphism (Bartoloni *et al.*, 2004). However, this polymorphism could be taken into consideration as a potential new marker for population genetic studies.

Discrepancies between results of association studies is a rather frequent phenomenon and may be related to many different factors (Ioannidis *et al.*, 2001). An important feature of this study is that the discrepancy between our analysis and the Chinese study is not related to sampling biases (small sample size, inadequate control group or population sub-structuring), but it is related to the complete absence of the 'at risk' SNP in 300 subjects, indicating that in Italy this polymorphism is probably absent or extremely rare. This remarkable difference is paradigmatic as it shows how ethnic background is important for polymorphisms involved in spermatogenesis, thereby underscoring that different genetic 'risk' factors may be present in different populations. A similar phenomenon is observed for cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations causing azoospermia because of congenital bilateral absence of vas deferens (CBAVD). The frequency of a particular CFTR mutation is also influenced by the ethnic composition of the population analysed (Patrizio & Leonard, 2000). Our study therefore contributes to a better definition of clinically relevant tests, specifically based on the ethnic origin of the infertile patients.

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