ORIGINAL ARTICLE

An ADP-ribosyltransferase 3 (*ART3*) variant is associated with reduced sperm counts in Czech males: case/control association study replicating results from the Japanese population

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Abstract

OBJECTIVES: In about 50% of male infertility the underlying pathogenesis remains unknown. A recent Japanese study provided evidence that the rs6836703: G>A single-nucleotide polymorphism (SNP) from the ADP-ribosyltransferase 3 (*ART3*) gene is significantly associated with non-obstructive azoospermia. However, the functional significance of this association is unknown and replication studies in unrelated populations are thus necessary.

DESIGN: In this study, 257 fertile Czech controls of proven paternity and 98 sub-/infertile patients selected according to stringent exclusion / inclusion criteria were genotyped by High Resolution Melting (HRM) of small amplicons.

SETTING: This study was performed at University Hospital Motol – Laboratory of reproductive genetics using routinely analyzed cases.

RESULTS: Significant differences in allele distribution between fertile and sub-/infertile men were found (OR=1.78, 95% CI: 1.17–2.70; p=0.007). Following sub-stratification of cases according to their sperm counts we found that observed differences in allele distributions were increased in oligozoospermic men with sperm counts of <15 million sperm/mL (OR=1.98, 95% CI: 1.28–3.07; p=0.002). This difference was also reflected in genotype distributions between fertile and sub-/infertile men (p=0.008), and fertile versus oligozoospermic men (p=0.004).

CONCLUSIONS: Our study serves as a first replication of the original Japanese report and opens new avenues of research. Compared to the Japanese patient cohort, we provided evidence that the analyzed *ART3* variant is associated with quantitative impairment of spermatogenesis.

Abbreviations:

ART3	- ADP-ribosyltransferase 3
ART	- Assisted Reproduction Treatment
ARTs	- mono-ADP-ribosyltransferases
AZF	- Azoospermia Factor
bp	- base pair
χ^2	- chi-square
DNA	- Deoxyribonucleic Acid
HapMap-CEU	- Haplotype Map of Utah residents with ancestry from Northern and Western Europe
HRM	- High Resolution Melting
HWE	- Hardy-Weinberg Equilibrium
NOA	- Non-Obstructive Azoospermia
OR	- Odds-Ratio
PCR	- Polymerase Chain Reaction
SNP	- Single-Nucleotide Polymorphism
WHO	- World Health Organization

INTRODUCTION

Infertility affects approximately 10–15% of couples in Western countries and in about 50% of male infertility cases the underlying pathogenesis has not been identified (Krausz & Giachini 2007; Ferlin *et al.* 2006). Considering the complexity of spermatogenesis and the increasing number of genes associated with this process, it is likely that molecular alterations in such genes are responsible, at least in part, for the unexplained cases of male infertility (Aston & Carrell 2009). Thus far, there have been multiple attempts to identify associated genetic risk factors (Krausz & Giachini 2007; Ferlin *et al.* 2006; Aston & Carrell 2009).

Recently, a Japanese report provided evidence that the rs6836703: G>A single-nucleotide polymorphism (SNP) of the ADP-ribosyltransferase 3 (*ART3*) gene located into the intron 10 (NCBI database, reference sequence NT016354.18, accessed by 05-25-2010), is significantly associated with non-obstructive azoospermia (NOA; p=0.027) (Okada *et al.* 2008). The *ART3* is a single-copy gene located on chromosome 4p15.1-p14, contains 11 exons and spans 33.13 kb of DNA (Glowacki *et al.* 2002; Friedrich *et al.* 2006b). The ART3 protein is a member of the mono-ADP-ribosyltransferases (ARTs) family (EC 2.4.2.31); they catalyze the reversible post-translational protein modification mono-ADP-ribosylation that can be used as a mechanism to regulate endogenous protein functions (Koch-Nolte 1997; Glowacki *et al.* 2002; Friedrich *et al.* 2006a). Currently, the best characterized ARTs are bacterial toxins, e.g. related to cholera or pertussis, which interfere with signal transduction by attachment of ADP-ribose onto regulatory G-proteins (Koch-Nolte *et al.* 2007; Glowacki *et al.* 2002; Friedrich *et al.* 2006a). However, the biological function of ART3 remains unclear since this protein lacks the active site motif (R-S-EXE) that is essential for the catalytic activity of arginine-specific transferases (Friedrich *et al.* 2006a; Friedrich *et al.* 2006b). In humans, the ART3 protein is expressed predominantly in spermatocytes, suggesting that it could play an important role in spermatogenesis (Friedrich *et al.* 2006a; Friedrich *et al.* 2006b; Okada *et al.* 2008).

The aim of this study is to replicate the Japanese observation in a representative cohort of Czech sub-/infertile males versus fertile controls in order to substantiate the association of the *ART3* rs6836703: G>A variant with impaired spermatogenesis and open new areas of research which may elucidate the role of *ART3* in spermatogenesis.

MATERIALS AND METHODS

A total of 257 males with proven paternity (i.e. fathered at least one child by natural conception) from a random cohort examined between 2003–2011 at our Department were genotyped for the rs6836703: G>A *ART3* variant. Their results were compared with corresponding data from 98 sub-/infertile men who underwent assisted reproduction treatment (ART) at our Center. Their clinical selection was in accordance with previously published exclusion criteria (Hucklenbroich *et al.* 2005; Wu *et al.* 2007; Yang *et al.* 2008). Patients with foreign origin and known causes of infertility were excluded (e.g. with varicocele, AZF microdeletions, orchitis, cryptorchidism, chromosomal aneuploidies associated with infertility such as aberrations of gonosomes – data not shown, obstructive azoospermia) (Hucklenbroich *et al.* 2005; Wu *et al.* 2007; Yang *et al.* 2008). The sub-/infertile group was further substratified into two groups according to the WHO classification (WHO 2010): a) azoospermic (n=18) and b) oligozoospermic (n=80, sperm counts <15 million sperm/mL). All cases and controls involved signed the informed consent that was approved by the Ethics Committee of the University Hospital Motol.

For genotyping we used the previously validated method High Resolution Melting (HRM) of small amplicons (Norambuena *et al.* 2009). Genomic DNA was extracted from leukocytes of peripheral blood using PUREGENE Genomic DNA Purification Kit (Gentra Systems, MN, USA) according to manufacturer's recommendations and stored at –20 °C. Before storage, all DNA samples were diluted to a concentration of 10 ng/μL using the PUREGENE™ DNA Hydratation Solution from Gentra Systems as dilution buffer, dilutions were kept at +4 °C. Primers were designed to amplify a 49 bp fragment around the SNP site and to avoid other sequence variations within the primer region (F: 5´-GTTGCTCTGGGTGGTGTTGAGC/R: 5´-ACAGTAGTGTCCCAGGCCTTCAC). PCR reaction was performed in a 10 μL reaction volume which consisted in 2 μL of genomic DNA (10 ng/μL) added to 8 μL of "reaction master mix" consisting of 1X LightCycler® 480 High Resolution Melting Master with 2.5 mM $MgCl_2$ (Roche Diagnostics, Germany) and 0.5 μM of forward and reverse primers. Rapid two-step PCR cycling, with an initial denaturation of 5 min at 95 °C continued by 35 cycles of 5 s at 95 °C and 25 s at 69 °C for annealing

and extension. Then, the program allowed one step for heteroduplex formation by heating to 95 °C for 10 s and cooling down to 40 °C for 5 s. For HRM, the plate was pre-heated at 72 °C for 10 s and afterward, heated from 72 °C to 95 °C performing 25 acquisitions per °C. The PCR reaction was performed on a 96-well plate in the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Germany).

For melting curve analysis all samples with late amplification, monitored by real time PCR and/or with a fluorescence <60% of the maximum value, were excluded (Norambuena *et al.* 2009). Following amplification and HRM respective normalized and normalized temperature-shifted difference plots for the rs6836703: G>A variant were analyzed. We were able to perform software-based genotype calls and visually differentiate the three expected melting profiles for each genotype group: G/G, G/A and A/A (Figure 1). HRM of small amplicons was our method of choice for the examination of the rs6836703: G>A SNP since it is a rapid, accurate and cost effective method (Norambuena *et al.* 2009).

Fertile controls are in conformity with Hardy-Weinberg equilibrium (HWE; non-significant results from Pearson's chi-square test (χ^2) calculated from rs1801133: C>T genotype/allele distributions; data available upon request) which marginalizes sampling bias. Association studies were analyzed by the odds-ratio (OR) and χ^2 (with Yates´s correction, where applicable), where *p*-values ≤0.05 were considered statistically significant.

Tab. 1. *ART3* rs6836703: G>A allele distribution.

	Allele		OR (95% CI)	*p*-value
	G	A		
Fertile	442/514 (0.860)	72/514 (0.140)		
Sub-/infertile men (all)	152/196 (0.776)	44/196 (0.224)	**1.78 (1.17-2.70)**	**0.007**
Azoospermic	31/36 (0.861)	5/36 (0.139)	0.99 (0.37-2.63)	1
Oligozoospermic	121/160 (0.756)	39/160 (0.244)	**1.98 (1.28-3.07)**	**0.002**

Tab. 2. *ART3* rs6836703: G>A genotype distribution.

	Genotype			
	G/G	G/A	A/A	*p*-value
Fertile	194/257 (0.755)	54/257 (0.210)	9/257 (0.035)	
Sub-/infertile men (all)	58/98 (0.592)	36/98 (0.367)	4/98 (0.041)	**0.008**
Azoospermic	13/18 (0.722)	5/18 (0.278)	0/18 (0.000)	0.939 (1)
Oligozoospermic	45/80 (0.563)	31/80 (0.388)	4/80 (0.050)	**0.004**

(1) Yate's correction

RESULTS

We observed a marked overall difference in allelic distribution between fertile and sub-/infertile Czech cohorts, since the frequency of allele "A" was significantly increased in infertile males (p=0.007; Table 1). This observation was also reflected in the distribution of G/A and A/A genotypes in sub-/infertile men (p=0.008; Table 2). When breaking down our aggregate results according to sperm counts (as specified above), we observed that allele "A" was particularly "enriched" in oligozoospermic men, i.e. in group b), compared to fertile controls (p=0.002; Table 1).

DISCUSSION

In this study we have replicated the significant association between the *ART3* rs6836703: G>A variant originally detected in the Japanese population in an unrelated Czech cohort. The "A" allele was comparatively "enriched" in oligozoospermic men, while the corresponding reduction of the allele "G" in infertile cases indirectly supports the "protective effect" of this allele on spermatogenesis.

Our observation of the increase of allele "A" frequencies in cases versus controls is not skewed due to insufficient sample size and/or sampling bias as verified by non significant deviation from the Hardy-Weinberg equilibrium in control samples. Given the low allele frequency for the "A" allele in the *ART3* variant rs6836703: G>A, we calculated HWE equilibrium from rs1801133: C>T (MTHFR c.677C>T; p.A222V) genotype/allele distributions which has a higher frequency for the recessive allele (data available upon request) minimizing type I error which is the probability of rejecting a true null hypothesis leading to false exclusion of associated markers, usually disease-associated SNPs, in HWE calculations. Tests for HWE presume that genotypes are randomly collected from the general population. In most association studies controls are selected by their apparently health status, thus being relatively "over-represented" compared to the general population. This could be a compounding feature for common diseases but is not applicable to our case (Salanti *et al.* 2005; Li & Li 2008; Wang & Shete 2010). In addition, potential of false positivity in HRM of small amplicons is negligible (Liew *et al.* 2004; Norambuena *et al.* 2009; Wittwer 2009).

Absence of association with azoospermia (Tables 1 and 2) may indicate that the *ART3* variant rs6836703: G>A likely causes a milder, i.e. "quantitative", reduction in spermatogenesis within the Czech population. However, we did not find any homozygous for the "A" allele within the azoospermic cohort most likely due to the lower number of azoospermic patients (n = 18) who

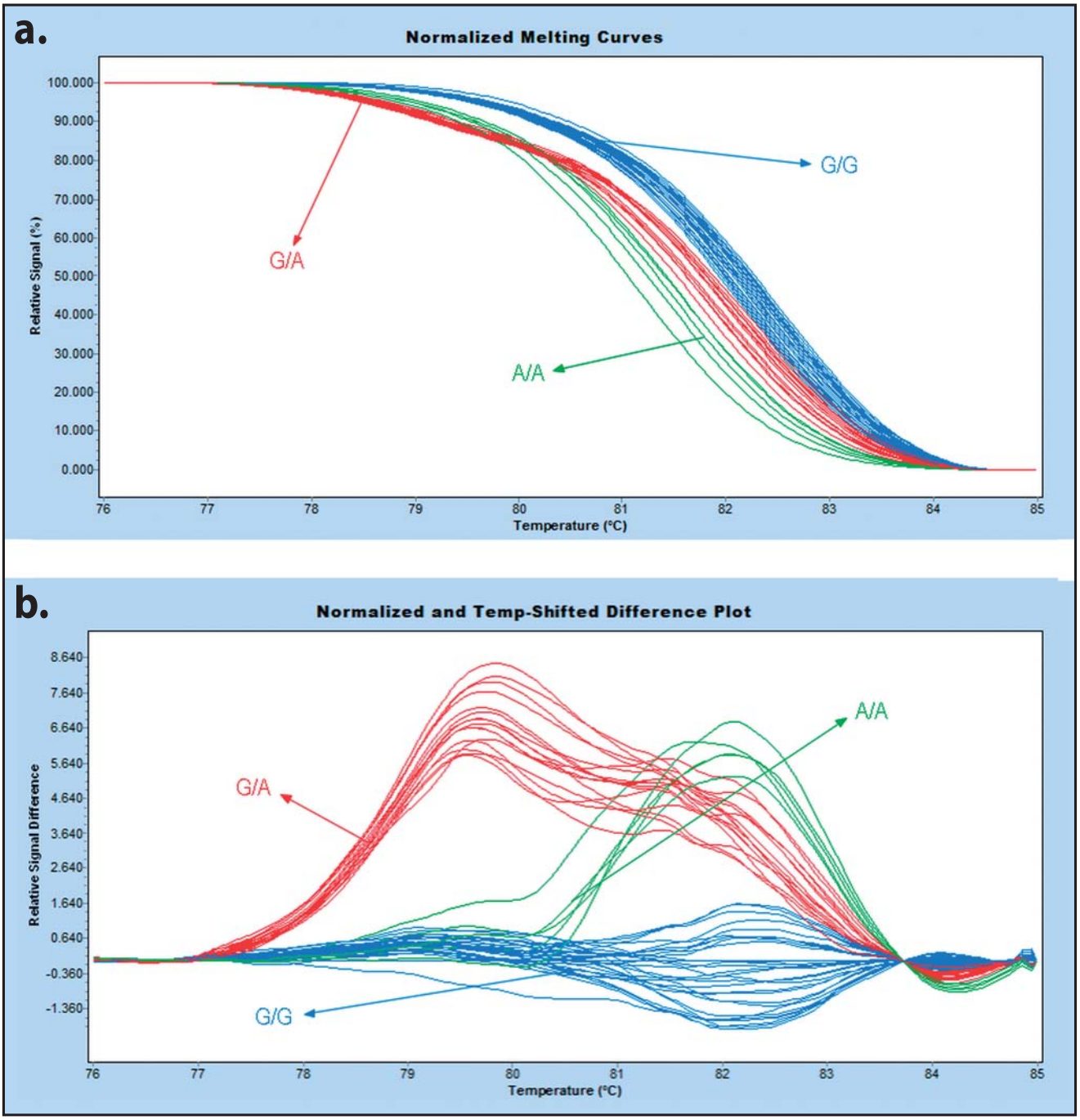

Fig. 1. Genotyping of rs6836703: G>A SNP by HRM of small amplicons. From the normalized melting curves (panel "a") it is possible to recognize both homozygous groups (G/G and A/A) by the difference in their melting temperature (Tm) and the heterozygous group (G/A) by the difference in the melting curve shape. Normalized temperature-shifted difference plot (panel "b") helps to the assignment by eye-inspection increasing the difference of the shape between melting profile groups. Control samples for each genotype (G/G, G/A and A/A) were run in duplicate. Each genotype group is indicated by arrows.

were selected according to stringent exclusion / inclusion criteria (Hucklenbroich *et al.* 2005; Wu *et al.* 2007; Yang *et al.* 2008) from a larger initial cohort of infertile cases. Moreover, there is a particularly low frequency of the "A/A" genotype in the European population (HapMap-CEU: G/G=0.750; G/A=0.250; A/A=0.0; dbSNP accesed by 09-19-2011) which further substantiates its potentially negative evolutionary selection.

It needs to be noted that the Japanese study included only patients with "non-obstructive azoospermia" without any further sub-stratification of cases according to their sperm counts by inclusion of sub-fertile patients (Okada *et al.* 2008). Moreover, the Japanese study group excluded patients with AZF microdeletions who could create, as in the Czech patient cohort, a strong confounding variable. We presume that observed inter-population differences might also reflect alternative

study designs (multiple variant versus single variant testing), differences in cohort sizes and/or the role of ethnic background / environmental factors (Tüttelmann *et al.* 2007; Aston & Carrell 2009).

Furthermore, the ethnic background of case-control studies needs to be taken into account. For instance, the previously reported association between a moderate expansion in the CAG repeats in the androgen receptor and reduced spermatogenesis in Asian populations has not been replicated in European studies (Ferlin *et al.* 2006; Rajender *et al.* 2007). Thus, in this study replication of original Japanese findings in an unrelated Czech cohort markedly increases the likelihood of its "real" functional association.

To date there is no further information on the pathway that disrupts spermatogenesis which could involve the rs6836703: G>A and/or ART3 protein (Glowacki *et al.* 2002; Friedrich *et al.* 2006a; Friedrich *et al.* 2006b; Okada *et al.* 2008). No difference was observed in testicular ART3 protein expression among haplogroups containing the rs6836703: G>A variant (Okada *et al.* 2008). The rs6836703: G>A variation might affect the expression/regulation of ART3 and/or of another protein(-s). In this regard, possible DNA-protein binding-sites for TFII-I, E2F-1 and PEA3 transcription factors were found for the DNA sequence containing the observed variation in *ART3* (PROMO – online program; Messeguer *et al.* 2002; Farré *et al.* 2003). Such *in silico* predictions may provide leads into additional studies of function of the ART3 protein or its DNA sequence in spermatogenesis. In any case, further replication studies in unrelated European-derived populations (Lao *et al.* 2008) are necessary in order to corroborate this difference. Eventual positive associations detected in unrelated populations could provide additional evidence for utilization of the respective *ART3* variant in reproductive genetics diagnostics.

In summary, our pilot study provided the first replication of the original Japanese report which suggested that *ART3* rs6836703: G>A variant is implicated in oligospermia.

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REFERENCES

1 Aston KI, Carrell DT (2009). Genome-wide study of single-nucleotide polymorphisms associated with azoospermia and severe oligozoospermia. J Androl. **30:** 711–725.
2 Farré D, Roset R, Huerta M, Adsuara JE, Roselló L, Albà MM, Messeguer X (2003). Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res. **31:** 3651–3653.
3 Ferlin A, Arredi B, Foresta C (2006).Genetic causes of male infertility. Reprod Toxicol. **22:** 133–141.
4 Friedrich M, Grahnert A, Paasch U, Tannapfel A, Koch-Nolte F, Hauschildt S (2006a). Expression of toxin-related human mono-ADP-ribosyltransferase 3 in human testes. Asian J Androl. **8**: 281–287.
5 Friedrich M, Grahnert A, Klein C, Tschöp K, Engeland K, Hauschildt S (2006b). Genomic organization and expression of the human mono-ADP-ribosyltransferase ART3 gene. Biochim Biophys Acta. **1759:** 270–280.
6 Glowacki G, Braren R, Firner K, Nissen M, Kühl M, Reche P, et al (2002). The family of toxin-related ecto-ADP-ribosyltransferases in humans and the mouse. Protein Sci. **11:** 1657–1670.
7 Hucklenbroich K, Gromoll J, Heinrich M, Hohoff C, Nieschlag E, Simoni M (2005). Partial deletions in the AZFc region of the Y chromosome occur in men with impaired as well as normal spermatogenesis. Hum Reprod. **20:** 191–197.
8 Koch-Nolte F, Haag F, Braren R, Kühl M, Hoovers J, Balasubramanian S, et al (1997). Two novel human members of an emerging mammalian gene family related to mono-ADP-ribosylating bacterial toxins. Genomics. **39:** 370–376.
9 Krausz C, Giachini C (2007). Genetic risk factors in male infertility. Arch Androl. **53**: 125–133.
10 Lao O, Lu TT, Nothnagel M, Junge O, Freitag-Wolf S, Caliebe A, et al (2008). Correlation between genetic and geographic structure in Europe. Curr Biol. **18**: 1241–1248.
11 Li M, Li C (2008). Assessing departure from Hardy-Weinberg equilibrium in the presence of disease association. Genet Epidemiol. **32:** 589–599.
12 Liew M, Pryor R, Palais R, Meadows C, Erali M, Lyon E, et al (2004). Genotyping of Single-Nucleotide Polymorphisms by High-Resolution Melting of Small Amplicons. Clin Chem. **50:** 1156–1164.
13 Messeguer X, Escudero R, Farré D, Núñez O, Martínez J, Albà MM (2002). PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics. **18:** 333–334.
14 Norambuena PA, Copeland JA, Krenková P, Stambergová A, Macek M Jr (2009). Diagnostic method validation: High resolution melting (HRM) of small amplicons genotyping for the most common variants in the MTHFR gene. Clin Biochem. **42:** 1308–1316.
15 Okada H, Tajima A, Shichiri K, Tanaka A, Tanaka K, Inoue I (2008). Genome-wide expression of azoospermia testes demonstrates a specific profile and implicates ART3 in genetic susceptibility. PLoS Genet.
16 Rajender S, Singh L, Thangaraj K (2007). Phenotypic heterogeneity of mutations in androgen receptor gene. Asian J Androl. **9:** 147–179.
17 Salanti G, Amountza G, Ntzani EE, Ioannidis JP (2005). Hardy-Weinberg equilibrium in genetic association studies: an empirical evaluation of reporting, deviations, and power. Eur J Hum Genet. **13:** 840–848.
18 Tüttelmann F, Rajpert-De Meyts E, Nieschlag E, Simoni M (2007). Gene polymorphisms and male infertility--a meta-analysis and literature review. Reprod Biomed Online. **15:** 643–658.
19 Wang J, Shete S (2010). Using both cases and controls for testing hardy-weinberg proportions in a genetic association study. Hum Hered. **69:** 212–218.
20 Wittwer CT (2009). High-resolution DNA melting analysis: advancements and limitations. Hum Mutat. **30:** 857–859.
21 World Health Organization (2010) WHO laboratory manual for the examination and processing of human semen 5th ed. Switzerland: WHO Press.
22 Wu B, Lu NX, Xia YK, Gu AH, Lu CC, Wang W, et al (2007). A frequent Y chromosome b2/b3 subdeletion shows strong association with male infertility in Han-Chinese population. Hum Reprod. **22**: 1107–1113.
23 Yang Y, Ma M, Li L, Zhang W, Chen P, Ma Y, et al (2008). Y chromosome haplogroups may confer susceptibility to partial AZFc deletions and deletion effect on spermatogenesis impairment. Hum Reprod. **23:** 2167–2172.