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Analysis of *DAZ* gene expression in a partial *AZFc* deletion of the human Y chromosome

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Abstract. The azoospermia factor c (*AZFc*) region of the Y chromosome consists of repetitive amplicons and is therefore highly susceptible to structural rearrangements, such as deletions and duplications. The b2/b3 deletion is a partial *AZFc* deletion that is conventionally determined by the selective absence of sY1191 in sequence-tagged site polymerase chain reaction (PCR) and is generally believed to retain two of the four deleted in azoospermia (*DAZ*) genes on the Y chromosome. In the present study we determined the copy number and expression of *DAZ* genes in sY1191-negative individuals. Using a *DAZ* dosage PCR assay and Southern blot analysis we evaluated the expression of four *DAZ* genes in five of six sY1191-negative individuals. Furthermore, cloning and immunoblot analyses revealed that three or more *DAZ* genes are expressed in sY1191-negative testes with germ cells. The results indicate that the selective absence of sY1191 not only means b2/b3 deletion with two *DAZ* genes, but also includes another *AZFc* configuration with four *DAZ* genes. These results exemplify the prevalence of variations in the *AZFc* region of the human Y chromosome.

Additional keywords: male infertility, microdeletion, testis.

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Introduction

The azoospermia factor c (*AZFc*) locus of the human Y chromosome contains massive sequence repeats called amplicons (Kuroda-Kawaguchi *et al.* 2001). The amplicons are organised in palindromic domains that harbour a cohort of genes for spermatogenesis (Kuroda-Kawaguchi *et al.* 2001). Because the ampliconic domains are highly susceptible to intra- and inter-chromosomal recombinations, microdeletions frequently occur in the *AZFc* region, often leading to spermatogenic failure in men (Kuroda-Kawaguchi *et al.* 2001; Skaletsky *et al.* 2003). For example, total deletion of *AZFc* results from recombination of the b2 and b4 domains, and spermatogenic failure is frequently observed in individuals with b2/b4 deletions (Reijo *et al.* 1995; Kuroda-Kawaguchi *et al.* 2001). The gr/gr deletion eliminates 1.6 Mb of sequence in the *AZFc* region and is considered to be a significant risk factor for impaired spermatogenesis (Repping *et al.* 2003). The b2/b3 deletion is another frequent deletion pattern in which an inversion and homologous recombination may occur sequentially within the *AZFc* locus (Fernandes *et al.* 2004; Repping *et al.* 2004). However, the effects of partial deletions on male fertility remain contentious. For example, some researchers have found an association between the b2/b3 deletion and infertility (Wu *et al.* 2007; Lu *et al.* 2009; Eloualid *et al.* 2012), whereas others have not (Repping *et al.* 2004; Hucklenbroich *et al.* 2005).

Sequence redundancy in *AZFc* makes it difficult to analyse the deletions with direct sequencing. Instead, unique sequence-tagged sites (STSs) at the *AZFc* region have been widely used to detect partial deletions within the region. For example, the gr/gr and b2/b3 deletions are determined in individuals with a selective absence of STS markers sY1291 and sY1191, respectively (Repping *et al.* 2003, 2004). The *AZFc* configuration resulting from these deletions has been further supported by the results of interphase fluorescent *in situ* hybridisation (FISH), Southern blot analyses and sequence family variant (SFV)-based assays (Fernandes *et al.* 2002, 2004; Repping *et al.* 2003, 2004; Lin *et al.* 2005, 2006). In this context, several association studies for the phenotypic consequences of partial *AZFc* deletions have relied largely on STS-PCR assays to detect such deletions (Hucklenbroich *et al.* 2005; Lynch *et al.* 2005; Ravel *et al.* 2006; Eloualid *et al.* 2012). However, this simple method may not provide information as to exactly which genes or gene copies are deleted in the chromosomes.

Deleted in azoospermia (*DAZ*) is a well known male infertility gene in the *AZFc* region of the Y chromosome (Saxena *et al.* 1996). *DAZ* and two autosomal homologues, namely deleted in

azoospermia-like (*DAZL*) and bol, boule-like (*Drosophila*) (*BOULE*), are critical for germ cell development in humans and other animals, such as mice, *Drosophila melanogaster* and *Caenorhabditis elegans* (Reijo *et al.* 1995; Eberhart *et al.* 1996; Ruggiu *et al.* 1997; Karashima *et al.* 2000; VanGompel and Xu 2010). The human *AZFc* region includes four *DAZ* genes with over 99% sequence identity (Saxena *et al.* 1996, 2000) and it has been shown that all the *DAZ* genes are actively transcribed and translated in the human testis (Kim *et al.* 2009). The DAZ proteins contain one or more RNA recognition motifs (RRMs) and multiple DAZ repeats (Yen *et al.* 1997; Saxena *et al.* 2000). It is of note that the DAZ proteins are polymorphic, containing different numbers of the RRMs and DAZ repeats (Kim *et al.* 2009).

By screening *AZFc* deletions in a previous study (Kim *et al.* 2009), we found a sample with a selective absence of sY1191 on STS-PCR that was indicative of the b2/b3 deletion. Interestingly, immunoblot analysis with a testicular sample revealed three discrete DAZ bands of different sizes (Kim *et al.* 2009). This result was unexpected because the model of the b2/b3 deletion predicts that two copies of *DAZ* are eliminated, leaving only two *DAZ* genes on the Y chromosome (Fernandes *et al.* 2004; Repping *et al.* 2004). In the present study we analysed the copy number of *DAZ* genes in sY1191-negative individuals and examined *DAZ* expression in their testes at the transcriptional and translational levels. The results unexpectedly revealed that most of the sY1191-negative individuals examined have more than two *DAZ* genes.

Materials and methods

Human samples and deletion screening of the AZFc *region*

All the subjects in the present study were ethnic Korean men who were recruited from an infertility clinic. Semen analyses and physical and clinical examination were performed according to conventional procedures performed in the clinic (Kim *et al.* 1999). In all, there were 150 subjects in the present study, comprising 148 infertile men who were diagnosed with either azoospermia or obstructive azoospermia and two fertile men (#001 and #155). Human samples for bloods and testes were obtained from all 150 subjects after they had provided informed consent following study approval by the Institutional Review Board of Seoul National University Hospital. All samples were stored at −80°C until analysis.

Genomic DNA was isolated from the blood according to the protocol described by Sambrook *et al.* (1989). Briefly, 10 mL fresh blood was centrifuged at 1300*g* for 15 min at 4°C and the buffy coat was obtained. The buffy coat was treated with 100 μg mL^{-1} proteinase K for 3 h. An equal volume of phenol equilibrated with Tris-HCl (pH 8) was added to the samples and gently mixed. The aqueous phase was collected after centrifugation at 5000*g* for 15 min at room temperature and mixed with an equal volume of chloroform. The aqueous phase was ethanol precipitated after mixing with 0.4 volumes of 5 M ammonium acetate. The precipitates were suspended with 10 mM TE8 and the DNA concentration was determined using a spectrophotometer.

The screening of *AZFc* deletions was conducted by STS-PCR with PCR conditions available at GenBank (http://www.ncbi.nlm.nih.gov/genbank/; accessed 9 December 2010) under the following accession numbers: sY142 (G38345), sY254 (G38349), sY255 (G65827), sY1191 (G73809), sY1197 (G67168), sY1201 (G67170), sY1206 (G67171), sY1291 (G72340). All STS-PCR were performed separately for each STS.

For the validation of the absence of a PCR product for sY1191, three different annealing temperatures (50, 56 and 61°C) were tested in the PCR for sY1191. In addition, two different pairs of primers were used to confirm the sY1191 deletion: (1) sY1191–2 (490 bp; tcagacactattttggcaatttt (forward) and ggcagcagaatcgcttgtat (reverse)), which targets the sY1191 locus and includes the original sY1191 targeting sequence of 385 bp in size; and (2) u3 (193 bp; o1276: agtctgagtggctagtgcac; o1277: gaagcaaagttcagctgtgc), which is part of u3 and close to sY1191 (Machev *et al.* 2004). A pair of PCR primers for *SRY* was also used as a control (Simoni *et al.* 2004). The PCR were performed using the following conditions: initial heating at 95°C for 15 min followed by 30 cycles of 94°C for 30 s, 50–61°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 4 min. The annealing temperatures were 50, 56 and 61°C for sY1191, 60°C for sY1191–2, 60°C for u3 and 55°C for *SRY*.

Reverse transcription–polymerase chain reaction

Testicular RNAs were extracted from human testicular samples using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNAs were reverse transcribed using random hexamers. The resultant cDNAs were amplified by PCR using the pairs of primers for the *DAZ* 3′-untranslated region (UTR), *DAZL*, *GAPDH* and the *DAZ* coding region. The pair of primers for the *DAZ* coding region was designed to amplify all four *DAZ* transcripts with one PCR reaction. The sequence information for the primers and the PCR conditions have been described in detail elsewhere (Kim *et al.* 2009).

Immunoblot analysis

Human testicular biopsy samples were homogenised in 1× sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting assays were conducted as previously described (Kim *et al.* 2009) with some modifications. Briefly, protein extracts were resolved on 10% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) for 30 min, and then incubated overnight with anti-DAZ antibody (1 : 100 diluted in blocking solution; Kim *et al.* 2009) at 4°C. After three washes with TBST for 10 min each time, membranes were incubated with a peroxidase-conjugated anti-rabbit IgG (1 : 10 000; Calbiochem, La Jolla, CA, USA) for 1 h at room temperature. The membranes were then washed another three times with TBST for 10 min each time, and peroxidase activity was detected using enhanced chemiluminescence (ECL) reagent. As a loading control, membranes were reprobed with anti-β-tubulin antibody (1 : 1000; Sigma, St Louis, MO, USA).

Assays to determine DAZ *dosage*

In the present study, we used the *DAZ*-dosage PCR assay developed and described by Lin *et al.* (2006) using primer pairs PrDAZ109 and PrDAZ110 and some modifications in the composition of PCR reaction mix. Briefly, the 10 μL PCR reaction volume contained 100 ng genomic DNA, 4 pmol each primers, 0.25 mM each dNTPs, 1 μL of 10× PCR buffer and 0.5 units Nova*clean*Taq polymerase (Genenmed, Seoul, Korea). The PCR was performed using the following conditions: initial heating at 95°C for 5 min followed by 25 cycles of 95°C for 20 s, 49°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR products were analysed on 2% agarose gels. Signal intensities were measured using ImageJ (National Institutes of Health (NIH), Besthesda, MA, USA).

Southern blot analysis

The probe for a dosage Southern blot was a mixture of two 1.4-kb PCR fragments containing the 3′ UTRs of *DAZ* and *DAZL*, and was prepared as described previously (Lin *et al.* 2006). The probe for Southern blot analysis of the RRM region of the *DAZ* genes was a 1.0-kb *Pst*I fragment containing the 3′ end of intron 1 of the *DAZ* genomic clone 7A69G (Lin *et al.* 2005).

Briefly, 3 μg genomic DNA was digested with 50 units *Nsi*I (restriction enzyme) for 6 h. The DNA fragments were separated on 0.5% agarose gels in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). Using denaturation/transfer solution (0.4 M NaOH, 0.8 M NaCl), the DNA fragments in the gels were denatured for 30 min and the transferred to nylon membranes for 7 h by capillary action with frequent towel changes. Membranes were soaked in neutralisation solution (0.5 M Tris-HCl, pH 7.2, 1 M NaCl) for 15 min and then baked at 80°C for 2 h. The membranes were then incubated for 1 h at 65°C in prehybridisation buffer (5× saline-sodium citrate (SSC), 5× Denhardt's reagent, 250 μg mL^{-1} salmon sperm DNA) and then hybridised with a radiolabelled probe overnight at 65°C in hybridisation buffer (5× SSC, 1× Denhardt's reagent, 250 μg mL^{-1} salmon sperm DNA, 10% dextran sulfate, 0.1% SDS). The DNA probes were ^{32}P labelled by random priming using the Prime-a-Gene labelling system (Promega, Madison, WI, USA) according to the manufacturer's instructions. At the end of hybridisation, the membranes were washed four times at 65°C with washing buffer (50 mM Tris, 1 mM EDTA, 1× Denhardt's reagent, 0.1% SDS, pH 8.0) over a 1 h period. After the final wash, the membranes were dried and exposed to a film at −80°C for 2 days. Signal intensities for a dosage Southern blot were measured using ImageJ (NIH).

Cloning and expression of DAZ *transcripts*

The PCR products amplified using primers for the *DAZ* coding region were used to clone *DAZ* transcripts from three sY1191-negative testicular cDNAs. After extraction with phenol–chloroform, the *DAZ* cDNAs were ligated into pGEM-T Easy Vector (Promega). The cloned *DAZ* transcripts were verified by sequence analyses. For mammalian expression of the *DAZ* cDNAs, the pGEM-T Easy Vector containing an entire protein coding region of *DAZ* was digested by *Eco*RI, and the resulting fragment was subcloned into *pcDNA3.1*(+) vector (Invitrogen). These constructs were subsequently transfected into 293T cells.

Cell culture and transfection

The culture of 293T cells and transient transfection using polyethylenimine have been described in detail elsewhere (Kim *et al.* 2009). Transfected cells were lysed and subjected to immunoblot analysis as described above.

Results

Screening for sY1191 deletion

To detect partial deletions in the *AZFc* locus, we performed genomic PCR analyses using specific STSs. We initially screened 150 subjects using three STSs (sY255, sY1191 and sY1291) and identified six individuals who lacked sY1191 only. We confirmed that the six sY1191-negative individuals had five additional STSs (sY142, sY254, sY1197, sY1201 and sY1206; Table 1). As controls, we included two individuals with an intact *AZFc*, two individuals with the sY1291-negative gr/gr deletions and two individuals with total *AZFc* deletions (Table 1; Kim *et al.* 2009).

The absence of a PCR product for sY1191 was further tested in order to validate that the result was due to genuine deletion rather than PCR failure. First, we examined the effect of lowered annealing temperatures in the PCR for sY1191, because non-optimal PCR conditions could result in false identification of deletion. Second, we used two different pairs of primers targeting the sY1191 locus (sY1191–2) and a sequence that is close to the sY1191 in the u3 region of *AZFc* (u3), because it is possible that STS-PCR failure may occur due to single nucleotide substitutions in primer sequences for STSs (Thornhill *et al.* 2002; Wu *et al.* 2011). In both tests, PCR fragments were amplified in the intact *AZFc* and gr/gr deletion samples, but not in the total *AZFc* deletion and six sY1191-negative samples (Fig. 1). These results strongly suggest that the sY1191-negative individuals have genuine deletions in the sY1191 region.

Expression of DAZ *in sY1191-negative testes*

To detect *DAZ* expression in *AZFc*-deleted testes, we performed reverse transcription–polymerase chain reaction (RT-PCR) analysis using a primer set targeting the 3′ UTR of all the *DAZ* cDNAs. A *DAZ*-specific band was detected in three of six sY1191-negative samples (Fig. 2*a*). In addition, the *DAZ*-specific band was detected in the intact *AZFc* and gr/gr deletion samples, but not in the total *AZFc* deletion sample (Fig. 2*a*). As a control, we determined the expression of *DAZL*, an autosomal homologue of *DAZ* with germ cell-specific expression (Yen *et al.* 1996). A *DAZL*-specific band was detected in all testicular samples except for the sY1191-negative samples that lacked the *DAZ*-specific band (Fig. 2*a*). The PCR fragment of *GAPDH* was detected in all samples examined (Fig. 2*a*). The RT-PCR results indicate that the sY1191-negative individuals without the *DAZ*- and *DAZL*-specific bands lack male germ cells in their testes (Fig. 2*a*). In support of this interpretation, the sY1191-negative individuals without the *DAZ*-specific bands were diagnosed

Table 1. Sequence-tagged site polymerase chain reaction analysis of the human samples used in the present study
STS, sequence-tagged site; +, positive; −, negative; ND, not determined; No, no deletion in the azoospermia factor c (*AZFc*) region; gr/gr^{del}, gr/gr deletion; *AZFc*^{del}, whole *AZFc* deletion; sY1191^{del}, sY1191-negative deletion

Individual	STS marker								Deletion type
	sY142	sY254	sY255	sY1191	sY1197	sY1201	sY1206	sY1291	
#119	+	+	+	+	+	+	+	+	No[A]
#146	ND	+	+	+	ND	ND	ND	+	No[A]
#001	+	+	+	+	+	+	+	−	gr/gr$^{del\ A}$
#105	ND	+	+	+	ND	ND	ND	−	gr/gr$^{del\ A}$
#077	+	−	−	−	+	+	−	−	*AZFc*$^{del\ A}$
#112	ND	−	−	−	ND	ND	ND	−	*AZFc*$^{del\ A}$
#033	+	+	+	−	+	+	+	+	sY1191^{del}
#108	+	+	+	−	+	+	+	+	sY1191^{del}
#124	+	+	+	−	+	+	+	+	sY1191^{del}
#113	+	+	+	−	+	+	+	+	sY1191^{del}
#147	+	+	+	−	+	+	+	+	sY1191^{del}
#155	+	+	+	−	+	+	+	+	sY1191^{del}

[A]Deletion type and *DAZ* expression were determined previously by Kim *et al.* (2009).

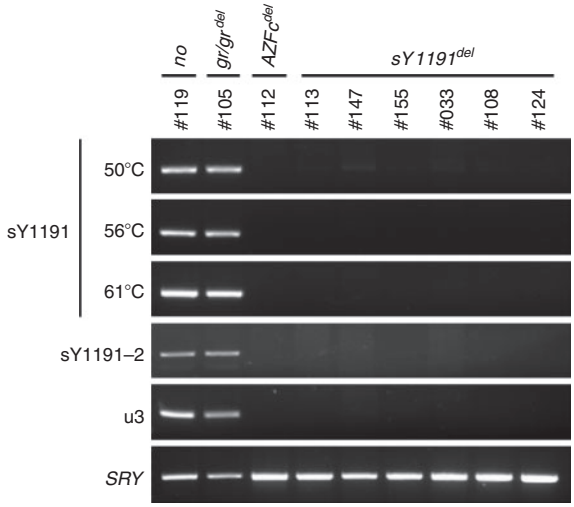

Fig. 1. Polymerase chain reaction (PCR) validation of sY1191 deletion. Genomic DNA was subjected to PCR amplification using primer sets that recognise the sY1191 locus (sY1191 and sY1191–2), a sequence of the u3 region near the sY1191 locus (u3) and sex-determining region Y (*SRY*) as a control. Three different annealing temperatures (50, 56 and 61°C) were used for sY1191 to test its optimality.

with azoospermia (#033, #108 and #124), whereas those with the *DAZ*-specific bands were diagnosed with obstructive azoospermia (#113 and #147) or normal (#155).

We then analysed DAZ protein expression in the sY1191-negative testes using a well-characterised DAZ antibody that detects both the DAZ and DAZL proteins in human testicular samples (Kim *et al.* 2009). As reported previously (Kim *et al.* 2009), four and two DAZ-specific bands were detected in the testicular samples with no *AZFc* deletion and gr/gr deletion, respectively (Fig. 2*b*). No DAZ-specific band was detected in the sample with a total *AZFc* deletion (Fig. 2*b*; Kim *et al.* 2009). Under our study conditions, three discrete DAZ bands were detected in the sY1191-negative samples that had germ cells (Fig. 2*b*). Consistent with the RT-PCR results, the DAZL-specific band was not detected in sY1191-negative samples without germ cells (Fig. 2*b*). Furthermore, the diverse DAZ-specific bands in the sY1191-negative samples confirmed the polymorphic nature of DAZ proteins (Fig. 2*b*; Kim *et al.* 2009). These results suggest that more than two *DAZ* genes are expressed in the sY1191-negative testes.

Determination of DAZ *copy numbers in sY1191-negative individuals*

We performed gene dosage assays to estimate the copy numbers of *DAZ* genes in individuals with a selective absence of sY1191. The autosomal *DAZL* gene was used as an internal dosage control for the determination of *DAZ* copy number. As comparative dosage controls, genomic samples with intact *AZFc* (#119; four *DAZ* genes), the gr/gr deletion (#105; two *DAZ* genes) and the *AZFc* deletion (#112; no *DAZ* gene) were used (Fig. 3). First, we determined the PCR band intensities of *DAZ* and *DAZL* using the six sY1191-negative subjects as well as the control samples, and compared their *DAZ*:*DAZL* signal ratios (Fig. 3*a*; Lin *et al.* 2006). Similarly, using dosage Southern blot, we determined *DAZ*:*DAZL* signal ratios in the same samples (Fig. 3*b*; Lin *et al.* 2006). Using both measures, the signal ratios of five sY1191-negative subjects (#113, #147, #155, #033 and #124) were similar to those of #119 with four *DAZ* genes; the exception was #108, which showed similar signal ratios as seen in #105 with two *DAZ* genes (Fig. 3). These results suggest that most of the sY1191-negative individuals examined have four *DAZ* genes.

Identification of DAZ *genes expressed in sY1191-negative testes*

To gain an insight into the structure of *DAZ* genes in the sY1191-negative individuals, we undertook Southern blot analysis of the

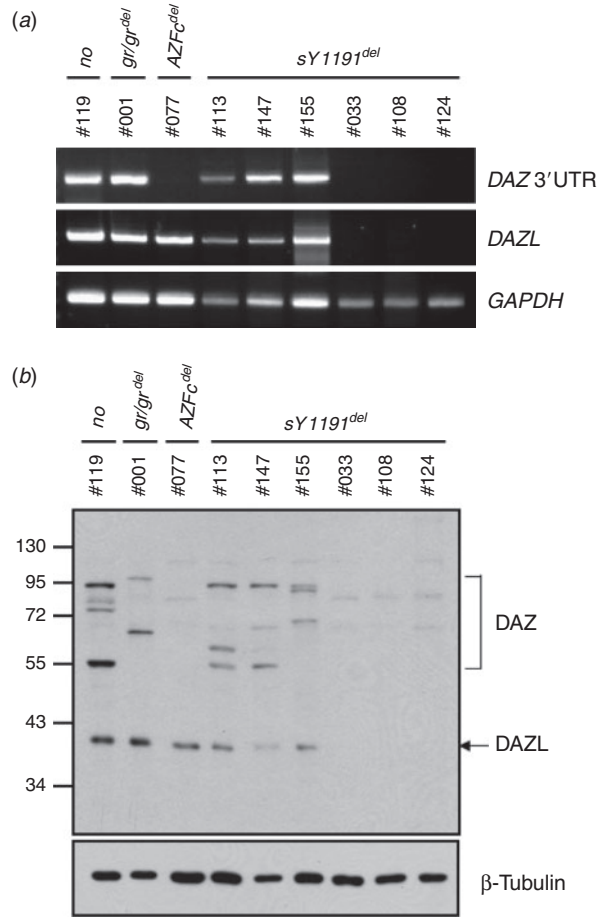

Fig. 2. Expression of deleted in azoospermia (*DAZ*) in testes with the azoospermia factor c (*AZFc*) deletion. (*a*) Reverse transcription–polymerase chain reaction analysis was performed with total RNA from testes of individuals with confirmed deletions at the *AZFc* locus. The RNAs were reverse transcribed and the resulting cDNAs were polymerase chain reaction amplified using primer sets for the *DAZ* 3′ untranslated region (UTR), *DAZL* and *GAPDH*. (*b*) Immunoblot analysis was performed with testis lysates from the same individuals. Note that the DAZ antibody recognises both the DAZ and deleted in azoospermia-like (*DAZL*) proteins in male germ cell lysates. The DAZ- and DAZL-specific bands are indicated. β-Tubulin served as a loading control.

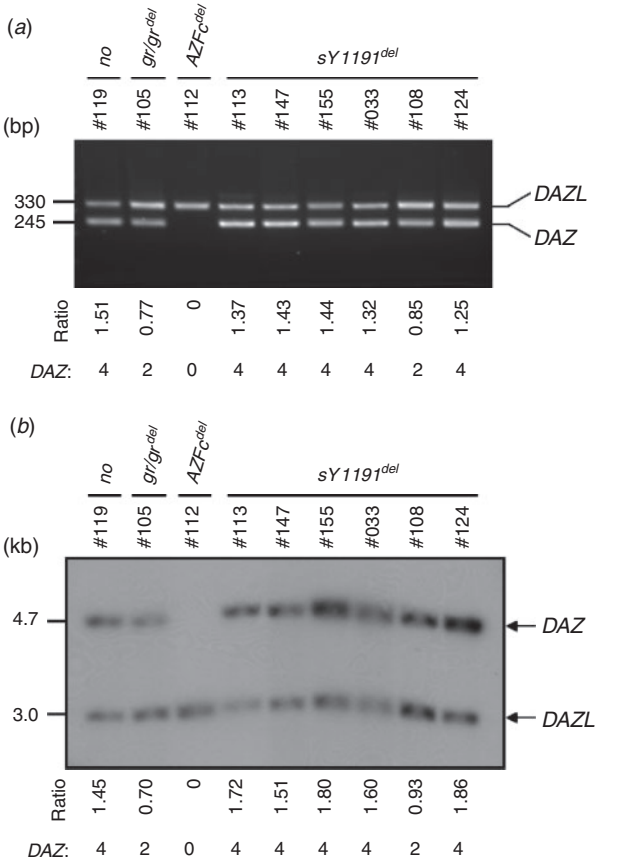

Fig. 3. Deleted in azoospermia (*DAZ*) dosage analyses. (*a*) Polymerase chain reaction (PCR) assay. Genomic DNA was subjected to PCR amplification using a primer set that recognises both *DAZ* and deleted in azoospermia-like (*DAZL*). The intensity ratio between the *DAZ*- and *DAZL*-specific bands and the corresponding *DAZ* copy number are shown at the bottom of the lanes. (*b*) Southern blot analysis. Genomic DNA digested with *Nsi*I was blotted using a probe for both *DAZ* and *DAZL*. The intensity ratio and *DAZ* copy number are shown. The copy number of *DAZ* genes is known for the first three (#119, #105, #112) samples (i.e. four (no deletion), two (gr/gr deletion) and zero (azoospermia factor c (*AZFc*) deletion), respectively).

RRM region of the *DAZ* genes. The probe used was designed to detect *Nsi*I fragments that are 31, 20 and 9 kb in size when the *DAZ* genes contain three, two and one RRM, respectively (Lin *et al.* 2005). Consistent with our previous report (Kim *et al.* 2009), the #119 sample had a *DAZ* gene with three RRMs (*DAZ1*), a *DAZ* gene with two RRMs (*DAZ4*) and two *DAZ* genes with a single RRM (*DAZ2* and *DAZ3*), whereas the #105 sample had a *DAZ* gene with three RRMs (*DAZ4*) and a *DAZ* gene with a single RRM (*DAZ3*; Fig. 4). Hybridisation patterns of the six sY1191-negative subjects showed that three subjects (#113, #147 and #033) have at least three *DAZ* genes with three, two and one RRM each, whereas the others (#155, #108 and #124) have at least two *DAZ* genes with three and one RRM

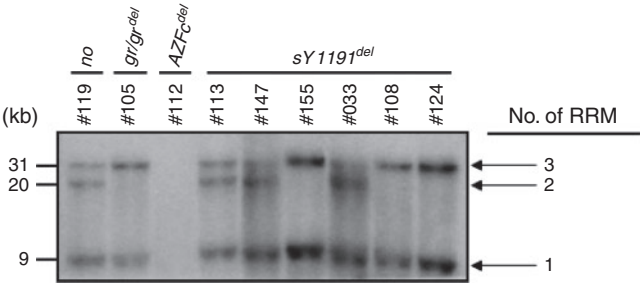

Fig. 4. Southern blot analysis of the RNA recognition motif (RRM) region of the deleted in azoospermia (*DAZ*) genes. Genomic DNA was digested with *Nsi*I and hybridised with a probe specific to the RRM regions. The number of RRMs in a specific *DAZ* band is indicated on the right, with fragments of 31, 20 and 9 kb corresponding to three, two and one RRM, respectively. The *DAZ* gene structures of #119 and #105 have been determined previously (Kim *et al.* 2009).

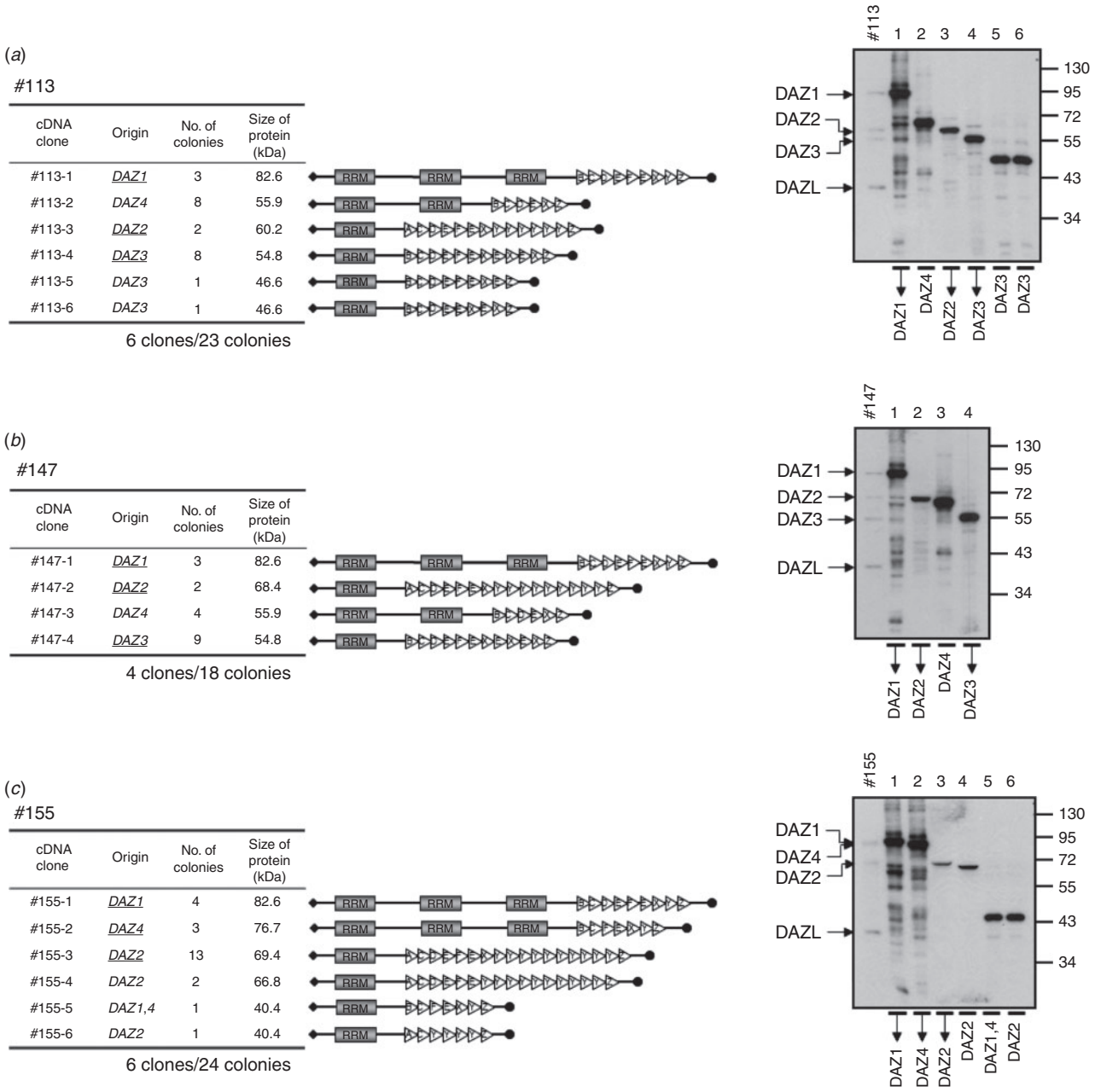

Fig. 5. Identification of deleted in azoospermia (*DAZ*) genes expressed in the sY1191-negative testes. The *DAZ* cDNAs were cloned from sY1191-negative testes, namely #113 (*a*), #147 (*b*) and #155 (*c*). The *DAZ* clones are depicted with the start and stop codons (dots), the RNA recognition motifs (RRMs; boxes) and DAZ repeats (triangles). Subtypes of the DAZ repeats are indicated inside the triangle. Immunoblot analysis of DAZ was performed using human testis lysates along with the *DAZ* cDNA clones expressed in 293T cells by transient transfection. By comparing the protein sizes, we identified the cDNA clones matching the DAZ proteins expressed in human testes (underlined).

(Fig. 4). This suggests that the numbers and kinds of *DAZ* genes in sY1191-negative subjects are heterogeneous.

To identify which *DAZ* genes are expressed in the sY1191-negative individuals, we cloned the *DAZ* transcripts expressed in the three sY1191-negative testes with germ cells. We performed RT-PCR with primers targeting the full-length coding region of *DAZ* and isolated the multiple PCR fragments that ranged from 2 to 4 kb (data not shown). These PCR fragments were then cloned and sequenced. As reported previously (Kim *et al.* 2009), this method enables us to identify the original *DAZ* transcripts in subjects, but cannot avoid derivatives resulting from PCR artefacts due to the repetitive nature of *DAZ* genes. Therefore, we determined a cDNA clone as the real transcript from a specific *DAZ* gene rather than a PCR artefact as follows: (1) the origins of the *DAZ* cDNAs were sorted out, based on the sequence characteristics of the *DAZ* copies (Fig. 5; Saxena

et al. 2000; Fernandes *et al.* 2002); and (2) the largest clone of a specific *DAZ* gene was counted as the original transcript and the smaller clones were considered artefacts with deletions during PCR amplification (Yen *et al.* 1997; Kim *et al.* 2009). We observed that all the *DAZ* cDNA clones originated from three or four different *DAZ* genes in each sY1191-negative testicular sample (Fig. 5). The *DAZ* cDNAs in the #113 and #147 samples originated from *DAZ1*, *DAZ2*, *DAZ3* and *DAZ4*, whereas those in the #155 sample originated from *DAZ1*, *DAZ2* and *DAZ4* (Fig. 5). The #113 and #147 samples included the original *DAZ* transcripts with one, two and three RRMs, whereas the #155 sample included the *DAZ* transcripts with only one and three RRMs (Fig. 5). Of note, these results are consistent with the Southern blot analysis of the RRM region in Fig. 4.

We then identified the *DAZ* transcripts that were actually expressed in the testis. The *DAZ* cDNA clones were expressed ectopically in 293T cells, and their sizes were compared with the endogenous DAZ proteins of the same testicular samples. Among the *DAZ* cDNA clones isolated, we found that three of the clones matched the DAZ-specific bands in each testicular sample (Fig. 5). In the #113 and #147 testes, expression of DAZ1, DAZ2 and DAZ3 was detected; we could not detect endogenous DAZ4 proteins although *DAZ4* transcripts were identified in both cases (Fig. 5*a*, *b*). Consistent with the cloning data, DAZ1, DAZ2 and DAZ4 were expressed in the #155 testis (Fig. 5*c*). Interestingly, the testicular DAZ protein always matched the largest clone of each of three *DAZ* genes (Fig. 5), suggesting that the largest clone is the genuine transcript of each *DAZ* gene. Together, these results indicate that at least three or more *DAZ* genes are expressed in the sY1191-negative testes.

Discussion

In the present study, we determined the *DAZ* copy number in Y chromosomes with selective deletion of the sY1191 STS marker. The gene dosage assays predicted that one subject (#108) has two *DAZ* genes, whereas the remaining five subjects (#113, #147, #155, #033 and #124) have four *DAZ* genes. Similar results have been reported in a study in the Taiwanese population (Lin *et al.* 2007). Using expression analysis, we further confirmed that more than two *DAZ* genes with polymorphic structures are indeed expressed in the three sY1191-negative testes. Together, these results suggest that the selective absence of sY1191 not only means b2/b3 deletion with two *DAZ* genes, but also includes another *AZFc* configuration with four *DAZ* genes.

The b2/b3 deletion model for selective absence of sY1191 has been supported by FISH analysis (Repping *et al.* 2004). The same report also suggested that subsequent duplication in the b2/b3 deletion would restore the copy number of *DAZ* genes to four while retaining sY1191 negativity (Repping *et al.* 2004). The results of RRM Southern blot analysis in the present study suggest that, of the five sY1191-negative subjects with four *DAZ* genes, #124 could carry such a b2/b3 deletion–duplication. However, our results do not support that the remaining four subjects with four *DAZ* genes (#113, #147, #155 and #033) underwent the b2/b3 deletion and subsequent duplication, because they have three or more different *DAZ* genes rather than two types of *DAZ* genes. Thus, it is possible that non-homology-based recombination may contribute to the *AZFc* partial deletions in our subjects (for a review, see Navarro-Costa *et al.* 2010). The most striking example may be the possible presence of the *DAZ* genes on the short arm of the Y chromosome (Premi *et al.* 2010). Alternatively, it is speculated that sister chromatid exchanges following the deletion–duplication enabled sY1191-negative subjects to have four different *DAZ* genes (Lin *et al.* 2005, 2007).

We determined the expression of DAZ proteins in the three sY1191-negative subjects with male germ cells and observed at least three different kinds of DAZ proteins. The cDNA cloning results revealed that the #113 and #147 subjects express four *DAZ* transcripts. However, we failed to detect DAZ4 protein, probably because the DAZ4 band may be too weak to be detected under our immunoblot conditions. In samples from #155, we detected three *DAZ* transcripts and the corresponding proteins. In any case, we observed the presence of more than two *DAZ* genes, which indicates that sY1191 negativity is not confined to the b2/b3 deletion with two *DAZ* genes.

Several association studies have relied on the selective absence of sY1191 to identify b2/b3 deletions in large populations (Hucklenbroich *et al.* 2005; Wu *et al.* 2007; Lu *et al.* 2009; Eloualid *et al.* 2012). However, our results suggest that the selective absence of sY1191 represents heterogeneous *AZFc* configurations with different gene copy numbers. In this regard, the uncertainty of associations between b2/b3 deletion and male infertility may be due, in part, to heterogeneous *AZFc* structures of the sY1191-negative subjects used in previous studies. Large-scale variations in the *AZFc* region are widespread across human Y chromosome lineages (Repping *et al.* 2006). Our results also show the prevalence of variations in the *AZFc* region with respect to *DAZ* genes. Therefore, it is necessary to characterise the detailed organisation of the *AZFc* region in sY1191-negative subjects by developing and identifying deletions with novel amplicon-specific sequence markers (Navarro-Costa *et al.* 2007).

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References

Eberhart, C. G., Maines, J. Z., and Wasserman, S. A. (1996). Meiotic cell cycle requirement for a fly homologue of human *Deleted in Azoospermia*. *Nature* **381**, 783–785. doi:10.1038/381783A0

Eloualid, A., Rhaissi, H., Reguig, A., Bounaceur, S., El Houate, B., Abidi, O., Charif, M., Louanjli, N., Chadli, E., Barakat, A., Bashamboo, A., McElreavey, K., and Rouba, H. (2012). Association of spermatogenic failure with the b2/b3 partial *AZFc* deletion. *PLoS One* **7**, e34902. doi:10.1371/JOURNAL.PONE.0034902

Fernandes, S., Huellen, K., Goncalves, J., Dukal, H., Zeisler, J., Rajpert De Meyts, E., Skakkebaek, N. E., Habermann, B., Krause, W., Sousa, M., Barros, A., and Vogt, P. H. (2002). High frequency of *DAZ1/DAZ2* gene deletions in patients with severe oligozoospermia. *Mol. Hum. Reprod.* **8**, 286–298. doi:10.1093/MOLEHR/8.3.286

Fernandes, S., Paracchini, S., Meyer, L. H., Floridia, G., Tyler-Smith, C., and Vogt, P. H. (2004). A large *AZFc* deletion removes *DAZ3/DAZ4* and nearby genes from men in Y haplogroup N. *Am. J. Hum. Genet.* **74**, 180–187. doi:10.1086/381132

Hucklenbroich, K., Gromoll, J., Heinrich, M., Hohoff, C., Nieschlag, E., and 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. doi:10.1093/HUMREP/DEH558

Karashima, T., Sugimoto, A., and Yamamoto, M. (2000). *Caenorhabditis elegans* homologue of the human azoospermia factor *DAZ* is required for oogenesis but not for spermatogenesis. *Development* **127**, 1069–1079.

Kim, B., Lee, Y., Kim, Y., Lee, K. H., Chun, S., Rhee, K., Seo, J. T., Kim, S. W., and Paick, J. S. (2009). Polymorphic expression of DAZ proteins in the human testis. *Hum. Reprod.* **24**, 1507–1515. doi:10.1093/HUMREP/DEP032

Kim, S. W., Kim, K. D., and Paick, J. S. (1999). Microdeletions within the azoospermia factor subregions of the Y chromosome in patients with idiopathic azoospermia. *Fertil. Steril.* **72**, 349–353. doi:10.1016/S0015-0282(99)00255-1

Kuroda-Kawaguchi, T., Skaletsky, H., Brown, L. G., Minx, P. J., Cordum, H. S., Waterston, R. H., Wilson, R. K., Silber, S., Oates, R., Rozen, S., and Page, D. C. (2001). The *AZFc* region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat. Genet.* **29**, 279–286. doi:10.1038/NG757

Lin, Y. W., Thi, D. A., Kuo, P. L., Hsu, C. C., Huang, B. D., Yu, Y. H., Vogt, P. H., Krause, W., Ferlin, A., Foresta, C., Bienvenu, T., Schempp, W., and Yen, P. H. (2005). Polymorphisms associated with the *DAZ* genes on the human Y chromosome. *Genomics* **86**, 431–438. doi:10.1016/J.YGENO.2005.07.003

Lin, Y. W., Hsu, C. L., and Yen, P. H. (2006). A two-step protocol for the detection of rearrangements at the *AZFc* region on the human Y chromosome. *Mol. Hum. Reprod.* **12**, 347–351. doi:10.1093/MOLEHR/GAL038

Lin, Y. W., Hsu, L. C., Kuo, P. L., Huang, W. J., Chiang, H. S., Yeh, S. D., Hsu, T. Y., Yu, Y. H., Hsiao, K. N., Cantor, R. M., and Yen, P. H. (2007). Partial duplication at *AZFc* on the Y chromosome is a risk factor for impaired spermatogenesis in Han Chinese in Taiwan. *Hum. Mutat.* **28**, 486–494. doi:10.1002/HUMU.20473

Lu, C., Zhang, J., Li, Y., Xia, Y., Zhang, F., Wu, B., Wu, W., Ji, G., Gu, A., Wang, S., Jin, L., and Wang, X. (2009). The b2/b3 subdeletion shows higher risk of spermatogenic failure and higher frequency of complete *AZFc* deletion than the gr/gr subdeletion in a Chinese population. *Hum. Mol. Genet.* **18**, 1122–1130. doi:10.1093/HMG/DDN427

Lynch, M., Cram, D. S., Reilly, A., O'Bryan, M. K., Baker, H. W., de Kretser, D. M., and McLachlan, R. I. (2005). The Y chromosome gr/gr subdeletion is associated with male infertility. *Mol. Hum. Reprod.* **11**, 507–512. doi:10.1093/MOLEHR/GAH191

Machev, N., Saut, N., Longepied, G., Terriou, P., Navarro, A., Levy, N., Guichaoua, M., Metzler-Guillemain, C., Collignon, P., Frances, A. M., Belougne, J., Clemente, E., Chiaroni, J., Chevillard, C., Durand, C., Ducourneau, A., Pech, N., McElreavey, K., Mattei, M. G., and Mitchell, M. J. (2004). Sequence family variant loss from the *AZFc* interval of the human Y chromosome, but not gene copy loss, is strongly associated with male infertility. *J. Med. Genet.* **41**, 814–825. doi:10.1136/JMG.2004.022111

Navarro-Costa, P., Pereira, L., Alves, C., Gusmão, L., Proença, C., Marques-Vidal, P., Rocha, T., Correia, S. C., Jorge, S., Neves, A., Soares, A. P., Nunes, J., Calhaz-Jorge, C., Amorim, A., Plancha, C. E., and Gonçalves, J. (2007). Characterizing partial *AZFc* deletions of the Y chromosome with amplicon-specific sequence markers. *BMC Genomics* **8**, 342. doi:10.1186/1471-2164-8-342

Navarro-Costa, P., Gonçalves, J., and Plancha, C. E. (2010). The *AZFc* region of the Y chromosome: at the crossroads between genetic diversity and male infertility. *Hum. Reprod. Update* **16**, 525–542. doi:10.1093/HUMUPD/DMQ005

Premi, S., Srivastava, J., Epplen, J. T., and Ali, S. (2010). *AZFc* region of the Y chromosome shows singular structural organization. *Chromosome Res.* **18**, 419–430. doi:10.1007/S10577-010-9123-1

Ravel, C., Chantot-Bastaraud, S., El Houate, B., Mandelbaum, J., Siffroi, J. P., and McElreavey, K. (2006). GR/GR deletions within the azoospermia factor c region on the Y chromosome might not be associated with spermatogenic failure. *Fertil. Steril.* **85**, 229–231. doi:10.1016/J.FERTNSTERT.2005.07.1278

Reijo, R., Lee, T. Y., Salo, P., Allagappan, R., Brown, L. G., Rosenberg, M., Rozen, S., Jaffe, T., Straus, D., Hovatta, O., de la Chapelle, A., Silber, S., and Page, D. C. (1995). Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat. Genet.* **10**, 383–393. doi:10.1038/NG0895-383

Repping, S., Skaletsky, H., Brown, L., van Daalen, S. K., Korver, C. M., Pyntikova, T., Kuroda-Kawaguchi, T., de Vries, J. W., Oates, R. D., Silber, S., van der Veen, F., Page, D. C., and Rozen, S. (2003). Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. *Nat. Genet.* **35**, 247–251. doi:10.1038/NG1250

Repping, S., van Daalen, S. K., Korver, C. M., Brown, L. G., Marszalek, J. D., Gianotten, J., Oates, R. D., Silber, S., van der Veen, F., Page, D. C., and Rozen, S. (2004). A family of human Y chromosomes has dispersed throughout northern Eurasia despite a 1.8-Mb deletion in the azoospermia factor c region. *Genomics* **83**, 1046–1052. doi:10.1016/J.YGENO.2003.12.018

Repping, S., van Daalen, S. K., Brown, L. G., Korver, C. M., Lange, J., Marszalek, J. D., Pyntikova, T., van der Veen, F., Skaletsky, H., Page, D. C., and Rozen, S. (2006). High mutation rates have driven extensive structural polymorphism among human Y chromosomes. *Nat. Genet.* **38**, 463–467. doi:10.1038/NG1754

Ruggiu, M., Speed, R., Taggart, M., McKay, S. J., Kilanowski, F., Saunders, P., Dorin, J., and Cooke, H. J. (1997). The mouse *Dazla* gene encodes a cytoplasmic protein essential for gametogenesis. *Nature* **389**, 73–77. doi:10.1038/37987

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). 'Molecular Cloning: A Laboratory Manual.' (Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.)

Saxena, R., Brown, L. G., Hawkins, T., Alagappan, R. K., Skaletsky, H., Reeve, M. P., Reijo, R., Rozen, S., Dinulos, M. B., Disteche, C. M., and Page, D. C. (1996). The *DAZ* gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned. *Nat. Genet.* **14**, 292–299. doi:10.1038/NG1196-292

Saxena, R., de Vries, J. W., Repping, S., Alagappan, R. K., Skaletsky, H., Brown, L. G., Ma, P., Chen, E., Hoovers, J. M., and Page, D. C. (2000). Four *DAZ* genes in two clusters found in the *AZFc* region of the human Y chromosome. *Genomics* **67**, 256–267. doi:10.1006/GENO.2000.6260

Simoni, M., Bakker, E., and Krausz, C. (2004). EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions. State of the art 2004. *Int. J. Androl.* **27**, 240–249. doi:10.1111/J.1365-2605.2004.00495.X

Skaletsky, H., Kuroda-Kawaguchi, T., Minx, P. J., Cordum, H. S., Hillier, L., Brown, L. G., Repping, S., Pyntikova, T., Ali, J., Bieri, T., *et al.* (2003). The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* **423**, 825–837. doi:10.1038/NATURE01722

Thornhill, A. R., Guenther, A. J., Barbarotto, G. M., Session, D. R., Damario, M. A., Dumesic, D. A., and Snow, K. (2002). False-positive Y-microdeletion result for a fertile male caused by an alteration under a PCR primer. *Int. J. Androl.* **25**, 352–357. doi:10.1046/J.1365-2605.2002.00377.X

VanGompel, M. J., and Xu, E. Y. (2010). A novel requirement in mammalian spermatid differentiation for the DAZ-family protein Boule. *Hum. Mol. Genet.* **19**, 2360–2369. doi:10.1093/HMG/DDQ109

Wu, B., Lu, N. X., Xia, Y. K., Gu, A. H., Lu, C. C., Wang, W., Song, L., Wang, S. L., Shen, H. B., and Wang, X. R. (2007). A frequent Y chromosome b2/b3 subdeletion shows strong association with male infertility in Han-Chinese population. *Hum. Reprod.* **22**, 1107–1113. doi:10.1093/HUMREP/DEL499

Wu, Q., Chen, G. W., Yan, T. F., Wang, H., Liu, Y. L., Li, Z., Duan, S. W., Sun, F., Feng, Y., and Shi, H. J. (2011). Prevalent false positives of *azoospermia factor a* (*AZFa*) microdeletions caused by single-nucleotide polymorphism rs72609647 in the sY84 screening of male infertility. *Asian J. Androl.* **13**, 877–880. doi:10.1038/AJA.2011.51

Yen, P. H., Chai, N. N., and Salido, E. C. (1996). The human autosomal gene *DAZLA*: testis specificity and a candidate for male infertility. *Hum. Mol. Genet.* **5**, 2013–2017. doi:10.1093/HMG/5.12.2013

Yen, P. H., Chai, N. N., and Salido, E. C. (1997). The human *DAZ* genes, a putative male infertility factor on the Y chromosome, are highly polymorphic in the *DAZ* repeat regions. *Mamm. Genome* **8**, 756–759. doi:10.1007/S003359900560