# A simplified gene-specific screen for Y chromosome deletions in infertile men

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**Objective:** To test the diagnostic efficiency of a gene-specific, five-marker screening strategy for the detection of Y chromosome deletions.
**Design:** Prospective case study.
**Setting:** University genetics laboratory and reproductive clinics.
**Patient(s):** Six hundred twenty-seven infertile men and 212 fertile men.
**Intervention(s):** Peripheral blood samples were screened for Y chromosome deletions in a triple-blind fashion using three protocols: protocol I consisted of five gene-specific markers, including *USP9Y, DBY, SMCY, RBM1*, and *DAZ*; protocol II included 14 gene-specific markers; and protocol III consisted of six sequence-tagged sites (STSs) markers recommended by EAA/EMQN.
**Main Outcome Measure(s):** Deletion status of Y chromosome genes or sequence-tagged sites.
**Result(s):** Protocols I and II identified the same 41 infertile patients with Y deletions. Protocol III identified 38 infertile patients with Y deletions, and all 38 patients were also identified by protocols I and II. One patient with isolated *USP9Y* deletion and two patients with isolated *DBY* deletions, as detected by protocols I and II, could not be identified by protocol III.
**Conclusion(s):** We observed mostly consistent results between our protocols and the EAA/EMQN protocol. This gene-specific, five-marker screening panel provides the same diagnostic efficiency as the EAA/EMQN protocol and may be considered an alternative to the EAA/EMQN protocol. (Fertil Steril® 2007;87:1291–300. ©2007 by American Society for Reproductive Medicine.)

**Key Words:** Gene, Y deletion, male infertility

Genetic factors are frequently responsible for male infertility in humans. Of these genetic factors, numerical or structural chromosomal abnormalities, such as Klinefelter syndrome or chromosomal translocations, are well-documented causes of male infertility. Although the mechanisms are not completely understood, it was suggested that a variety of chromosomal anomalies exert an adverse effect on spermatogenesis, resulting in oligozoospermia or azoospermia (1, 2). More recently, deletions of AZoospermia Factors (AZFs) on the long arm of the Y chromosome (Yq) have been widely studied and recognized as the second most frequent genetic cause of spermatogenic failure after the Klinefelter's syndrome (3, 4). Based on observations of recurrent, nonoverlapping deletion patterns, it has been proposed that multiple genes on the Y chromosome might be implicated in spermatogenesis defects. These genes were located in the proximal, middle, and distal subregions of Yq11, designated as AZFa, AZFb, and AZFc, respectively (5).

Although Y chromosome deletions are causally related to spermatogenesis defects, significant differences exist in deletion frequency, location, and extent of deletion. In previous studies, the percentage of infertile men carrying Y deletions varied greatly, ranging from 1%–55% (6, 7). Moreover, the overall misdiagnosis rate of AZFb and AZFc deletions has been shown to be as high as 5%, and AZFc deletions have been identified in 3%–5% of men with a normal Y chromosome (8). These variations may have been the result of different study designs, patient selection, quality control of diagnostic laboratories, or genuine variations in the population (7, 9–11). In the literature, there is extreme heterogeneity in the panels of polymerase chain reaction (PCR) markers used by different laboratories to detect Y deletions. Presumably, the more markers used, the greater is the sen-

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sitivity of the screen. Using too many markers, however, might also lead to the detection of clinically irrelevant polymorphic variants and produce a labor-intensive and costly assay (12, 13). Given that Y chromosome deletion is an important genetic defect for infertile couples, it would be of value to develop a simplified and reliable test to detect these deletions in infertile men. The European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) have recommended a minimal set of primers for screening Y chromosome deletions, which includes six sequence-tagged sites (STSs) as a basic set of primers for the diagnosis: *sY84* and *sY86* for AZFa, *sY127* and *sY134* for AZFb, and *sY254* and *sY255* for AZFc, with *SRY* and *ZFY/ZFX* as controls. The EAA/EMQN best practice guidelines stated that this primer set enables the detection of all the clinically relevant deletions and of more than 95% of the deletions in the three AZF regions (14).

An alternative approach for Y chromosome deletion analysis is the use of gene-specific markers instead of anonymous STSs. The reason for this is that the use of gene-specific markers may contribute more information about the pathological role of the genes deleted. In our previous study, results from combined gene-specific marker screening and STS marker screening suggested that a set of *USP9Y* (ubiquitin-specific protease 9Y), *DBY* (Dead box Y), *RBM1* (RNA-binding motif Y), and *DAZ* (Deleted in azoospermia) would suffice to detect all cases with deletions in the Taiwanese population (13). Since then, information of the Y chromosome sequences has been published and nonallelic homologous recombination has been recognized as the deletion mechanism of Y chromosome (15–18). Based on this information, we have modified the four-maker screening panel to a five-marker panel, with the addition of *SMCY* (SMC mouse homolog Y), as a first-line screening tool in our laboratory.

In this study, we aimed to evaluate the diagnostic efficiency of this five-marker panel as a first-line screening for infertile men. We used a triple-blind study design that included three protocols. Protocol I consisted of five gene-specific markers, including *USP9Y, DBY, SMCY, RBM1*, and *DAZ*, which would presumably be the panel for first-line screening. Protocol II consisted of 14 gene-specific markers, which were used to verify protocol I and to determine the extensions of deletions. Protocol III consisted of six STSs recommended by EAA/EMQN, which could be used to compare the performances of our five-marker screening panel. We evaluated the protocol-to-protocol variances, analyzed the deletion patterns of our patients, and described the rationale for developing a simplified screen for AZF gene deletions.

## MATERIALS AND METHODS

### Subjects

The experimental design is in accord with the Helsinki Declaration of 1975 on human experimentation, and has been approved by the Institutional Review Board (IRB) of National Cheng Kung University Hospital. The signed informed consents have been obtained for all enrollees. From April 2001 to December 2005, a total of 627 infertile men presenting with oligozoospermia or nonobstructive azoospermia and 212 men with proven fertility (controls) were enrolled in this study. All subjects belonged to the Taiwanese Han, the major ethnic group in Taiwan. The 212 control subjects were recruited from husbands of women who received regular prenatal care at the University Hospital. They had normal chromosomal karyotype and had at least two children in 5 years without assisted reproductive technologies (ART). All infertile men enrolled had normal chromosomal karyotype and underwent a comprehensive examination, including a detailed history, physical examination, at least two consecutive semen analyses, and endocrinology profile (FSH, LH, PRL, and T). Testicular biopsy was recommended in patients without evident factors to differentiate between obstructive and nonobstructive azoospermia, and was performed as part of therapeutic process, such as testicular sperm extraction (TESE), in patients with clinical evidence of nonobstructive azoospermia (19). Nonobstructive azoospermia was defined as spermatogenic defects in the testicular biopsy (such as hypospermatogenesis, maturation arrest, and Sertoli cell-only syndrome), or elevated serum FSH level, total testicular volume less than 30 mL, and no other applicable diagnoses (20).

### Testing Strategies

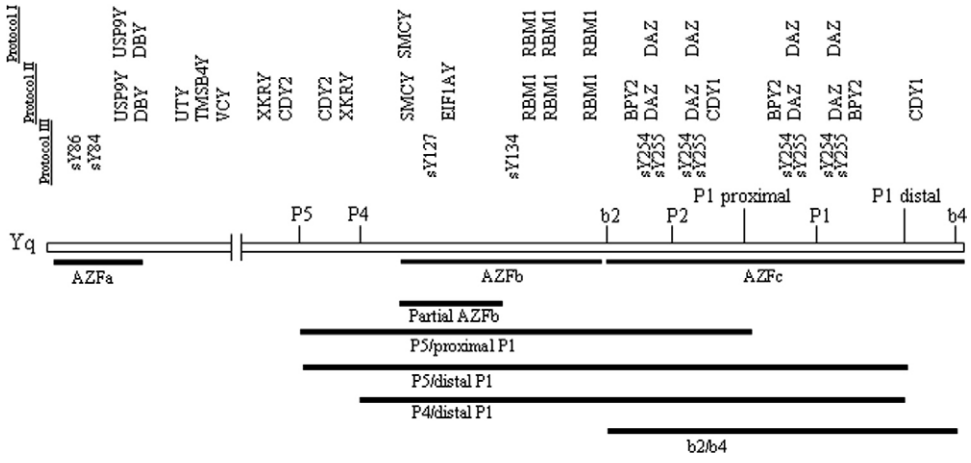
The molecular tests were performed using three protocols in a triple-blind design (Fig. 1), by three technicians who were blinded to the identity of the samples. Genomic DNA was extracted from peripheral blood samples using a Puregene DNA isolation kit (Gentra, Minneapolis, MN). The PCR was performed using primers specific to distinct genes or STSs and the products were amplified in multiplex fashion. All primer sequences are shown in Table 1. All PCR reactions were done using an OmniGene Thermal Cycler (Hybaid Ltd., Ashford Middlesex, United Kingdom). For each PCR assay, we incorporated the following samples as controls: genomic DNA from a normal fertile man, genomic DNA from a normal fertile woman, the plasmid DNA or a BAC clone, and a PCR mixture without template DNA (blank control). To confirm amplification failures by multiplex PCR, all markers were amplified by at least two single PCR reactions.

### Protocol I

Protocol I was based on two multiplex PCR reactions consisting of five gene-specific markers (*USP9Y, DBY, SMCY, RBM1*, and *DAZ*). The primer mixtures used in the multiplex PCR reactions were mixture A (*SRY, ZFY/ZFX, RBM1, SMCY*, and *DAZ*) and mixture B (*SRY, ZFY/ZFX, DBY*, and *USP9Y*). The *SRY* and *ZFY/ZFX* genes were used as internal controls. The PCR reactions consisted of 0.1 to 0.6 $\mu$M of each primer (0.1 $\mu$M for *SRY, RBM1, ZFY/ZFX*, 0.2 $\mu$M for *DBY*, 0.4 $\mu$M for *sY277*, 0.5 $\mu$M for *SMCY*, and 0.6 $\mu$M for *USP9Y*), 1 $\times$ PCR buffer, 1.5 mM $MgCl_2$, 200 $\mu$M of each dNTP, 150 ng of genomic DNA, and 2 units of *Taq* DNA polymerase (Perkin-Elmer Cetus

## FIGURE 1

Schematic representation of the Y chromosome map. From top to bottom: genes and STSs for the protocols I, II, and III, positions of palindromes (vertical lines), AZF locations proposed by Vogt et al. (black bars under Yq), and deletion patterns categorized by breakpoints of palindromes suggested by Kuroda-Kawaguchi et al., Repping et al. and Ferlin et al. (black bars).

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Corp., Emeryville, CA), in a total volume of 20 μL. The PCR amplification consisted of an initial denaturation at 95°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. A final extension step was carried out at 72°C for 10 minutes and the reaction products were fractionated on 2.5% agarose gels.

### Protocol II

Protocol II was based on methods described previously, with minor modifications (13). Briefly, 14 pairs of gene-specific primers located on Yq11.2 were used to amplify samples in four separate multiplex PCR reactions. Of the 14 gene-specific markers, two target genes are located in the AZFa region: *USP9Y* and *DBY*. Another six genes are located in AZFb and AZFc regions: *SMCY*, *EIF1AY* (Translation initiation factor 1A Y), and *RBM1* in AZFb, and *BPY2* (Basic protein Y 2), *DAZ*, and *CDY1* (Chromodomain Y 1) in AZFc. The last five target genes are located beyond the AZF region: *TMSB4Y* (Thymosin beta-4Y), *VCY* (variable charge Y), *UTY* (Ubiquitously TPR motif Y), *CDY2* (Chromodomain Y 2), and *XKRY* (XK related Y). Some genes locate within the AZF region were not included, such as the *HSFY, CYorf15A, CYorf15B, RPSY2, GSPG4LY, GALGA2LY*, and *TTY* gene families (15, 21). Although not all of the genes were screened, the application of 14 markers may provide a global view of the deletion status of potential AZFs. The four primer mixtures used in the multiplex PCR reactions were mixture I (*RBM1, SMCY*, and *CDY2*), mixture II (*DBY, sY283, USP9Y*, and *VCY*), mixture III (*XKRY, EIF1AY, CDY1*, and *UTY*), and mixture IV (*BPY2, sY277, TMSB4Y*). Sites *sY277* and *sY283* were specific to the *DAZ* gene; *SRY* and *ZFY/ZFX* act as the internal controls. To verify primer specificity, five BAC clones, each containing a single copy of *DAZ* (RP11-26D12), *CDY1* (RP11-497C14), *CDY2* (RP11-509B6), *BPY1* (RP11-264A13), or *BPY2* (RP11-86G22), and a plasmid containing partial sequences of the *RMB1* gene were used. The BAC clones were obtained from Pieter de Jong's "BACPAC Resources" group (http://www.chori.org/bacpac). The plasmid was kindly provided by Dr. Pauline Yen of the Institute of Biomedicine, Academia Sinica, Taiwan. Each protocol II PCR reaction consisted of 0.12–0.50 μM of each primer, 1 × PCR buffer, 1.50 mM $MgCl_2$ (for mixtures I, III, and IV) or 1.25 mM $MgCl_2$ (for mixture II), 200 μM of each dNTP, 150 ng of genomic DNA, and 2 units of *Taq* DNA polymerase (Perkin-Elmer Cetus Corp.), in a total volume of 20 μL. An initial denaturation step was carried out at 95°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. A final extension step was carried out at 72°C for 10 minutes and the reaction products were fractionated on 2.5% agarose gels (for mixtures I and II) or 12% acrylamide gels (for mixtures III and IV).

### Protocol III

Protocol III was based on methods of EAA/EMQN best practice guidelines (14), which includes *sY84, sY86* (AZFa), *sY127, sY134* (AZFb), *sY254, sY255* (AZFc), *SRY*, and *ZFY/ZFX*. Sites *sY254* and *sY255* are both in the *DAZ* gene. Two primer mixtures were used for PCR reactions according to EAA/EMQN guidelines: mixture I (*SRY, ZFY/ZFX, sY86,*

**TABLE 1**

**Primer sequences for protocols I, II, and III and for validation of AZFa deletion.**

| Gene/STS | Forward primer | Reverse primer | Position | Accession number |
|---|---|---|---|---|
| *SRY* | GAATATTCCCGCTCTCCGGA | GCTGGTGCTCCATTCTTGAG | 11–480 | G38356 |
| *ZFY/ZFX* | ACCRCTGTACTGACTGTGATTACAC | GCACYTCTTTGCTATCYGAGAAAGT | chrY: 2890344–chrY: 2890838 | |
| *USP9Y* | TTGAAGTTACTTTTATAATCTAATGCTT | ATAATTTATTACTTTACAGTCACAGTGG | 161442–161786 | AC002531 |
| *USP9Y-1* | GGCTGATATATGCTGGTACTTCATTCA | CAGTACTCAAAACAACACAG | 13268–13512 | AC002531 |
| *DBY* | ATCGACAAAGTAGTGGTTCC | AGATTCAGTTGCCCCACCAG | 67648–68336 | AC004474 |
| *DBY-1* | AGTTCCGCTATTCGGTCTCA | CCCTGAAGAGAAGCGAAAAA | 55085–55145 | AC004474 |
| *VCY* | CTCCCTGAGCAGCAACTAAG | GTCATCAACATGGGAAGCAC | 71584–71664 | NG_004442 |
| *UTY* | GCATCATAATATGGATCTAGTAG | GGGAGATACTGAATAGCATAGC | 1752–1817 | NM_007125 |
| *TMSB4Y* | CAAAGACCTGCTGACAATGG | CTCCGCTAAGTCTTTCACC | 1040–1141 | NM_004202 |
| *XKRY* | CACTCATGGAGAAGGGTAG | GTCACACTCAGCCTCTTTAC | 1413–1506 | AF000997 |
| *CDY2* | ACAGCCCCTTTGACCACAAG | TCTGCGACATTAGTGGGTGC | 761–958 | NM_001001722 |
| *SMCY* | CCTCCAGACCTGGACAGAAT | TGTGGTCTGTGGAAGGTGTCA | 71222–71584 | AF273841 |
| *EIF1AY* | CTCTGTAGCCAGCCTCTTC | GACTCCTTTCTGGCGGTTAC | 17–100 | AF000987 |
| *RBM1* | ATGCACTTCAGAGATACCGC | CCTCTCTCCACAAAACCAACA | 605990–606842 | NG_004755 |
| *BPY2* | GGGATTATCACATATTGCGG | ATGATAGTCGCGTCAGCTGG | 851–1157 | AF000980 |
| *sY277* (*DAZ*) | GGGTTTTGCCTGCATACGTAATTA | CCTAAAAGCAATTCTAAACCTCCAG | 3040645–3040956 | NG_004755 |
| *sY283* (*DAZ*) | CAGTGATACACTCGGACTTGTGT | AGTTATTTGAAAAGCTACACGGG | 3041874–3042371 | NG_004755 |
| *CDY1* | TGGGCGAAAGCTGACAGCA | AGGGTGAAAGTTCCAGTCAA | 1622–1699 | AF000981 |
| *sY86* | GTGACACACAGACTATGCTTC | ACACACAGAGGGACAACCCT | 21–338 | G49207 |
| *sY84* | GCCTACTACCTGGAGGCTTC | AGAAGGGTCTGAAAGCAGGT | 19636–19963 | AC004810 |
| *sY127* | GGCTCACAAACGAAAAGAAA | CTGCAGGCAGTAATAAGGGA | 58–331 | G11998 |
| *sY134* | ACCACTGCCAAAACTTTCAA | GTCTGCCTCACCATAAAACG | 107027–107329 | AC010086 |
| *sY254* | GGGTGTTACCAGAAGGCAAA | GAACCGTATCTACCAAAGCAGC | 7–386 | G38349 |
| *sY255* | GTTACAGGATTCGGCGTGAT | CTCGTCATGTGCAGCCAC | 17–139 | G65827 |
| *sY82* | ATCCTGCCCTTCTGAATCTC | CAGTGTCCACTGATGGATGA | 152–415 | G40972 |
| *sY83* | CTTGAATCAA AGAAGGCCCT | CAATTTGGTTTGGCTGACAT | 1–277 | G64734 |
| *sY85* | TGGCAATTTGCCTATGAAGT | ACAGGCTATTTGACTGGCAG | 121–489 | G49205 |
| *sY87* | TCTGTTGCTTGAAAAGAGGG | ACTGCAGGAAGAATCAGCTG | 47431–47681 | AC004474 |
| *sY88* | TTGTAATCCAAATACATGGGC | CACCCAGCCATTTGTTTTAC | 42–164 | G49210 |

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*sY127*, and *sY254*) and mixture II (*SRY, ZFY/ZFX, sY84, sY134*, and *sY255*). The PCR reactions consisted of 0.2 μM of each primer, 10 μL 2 × Qiagen Multiplex PCR Master-Mix (Qiagen, Hilden, Germany), 150 ng of genomic DNA, in a total volume of 20 μL. An initial denaturation step was carried out at 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 90 seconds, and extension at 72°C for 1 minute. A final extension step was carried out at 72°C for 10 minutes and the reaction products were fractionated on 2% agarose gels.

### Validation of Isolated AZFa Gene Deletions

A series set of STSs and another set of *USP9Y* and *DBY*-specific primers were used to verify the presence of isolated AZFa gene deletions. The gene-specific primers were designed according to the following principle: if the first set of primers was located in the 5′ end of the gene, the second set of primers would be located in the 3′ end, and vice versa. Seven STSs (*sY82, sY83, sY86, sY85, sY84, sY87*, and *sY88*) were used to define the extent of AZFa deletions. The PCR reaction consisted of 0.2 μM of each primer, 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 100 ng of genomic DNA, and 2 units of *Taq* DNA polymerase (Perkin-Elmer Cetus Corp.), in a total volume of 20 μL. An initial denaturation step was carried out at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 57°C (*sY86, sY84, USP9Y-1, sY87*) or 60°C (*sY82, sY83, sY85, DBY-1, sY88*) for 90 seconds, and extension at 72°C for 1 minute. A final extension step was carried out at 72°C for 10 minutes and the reaction products were fractionated on 1.5% agarose gels.

## RESULTS

### Patients

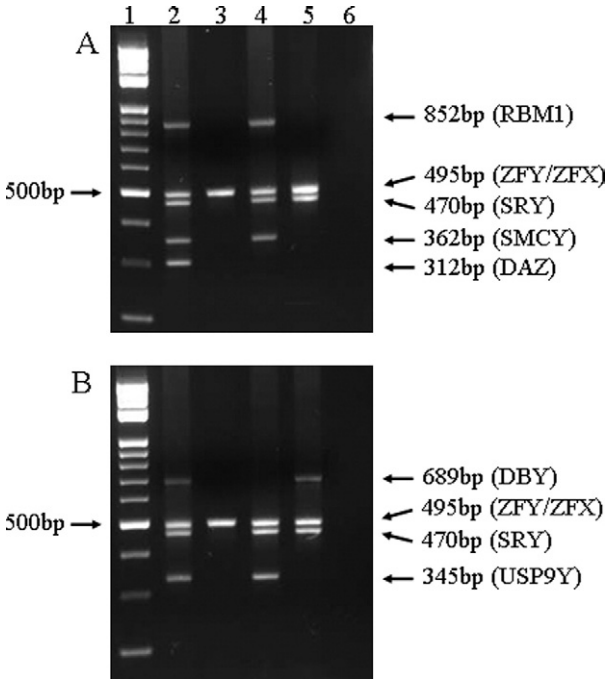
Of the 627 infertile men tested, 224 had idiopathic mild-to-moderate oligospermia (sperm counts between 5 × $10^6$/mL and 20 × $10^6$/mL), 110 had idiopathic severe oligospermia (sperm counts between 1 × $10^6$/mL and 5 × $10^6$/mL), 23 had virtual azoospermia (sperm counts <1 × $10^6$/mL), and 270 had nonobstructive azoospermia. Of the 270 men with nonobstructive azoospermia, testicular histopathological diagnoses were obtained in 181 men who received testicular biopsy or TESE. Of these, 81 were diagnosed with hypospermatogenesis, 24 with maturation arrest, and 76 with Sertoli cell-only syndrome.

### Deletions Detected Using Protocol I

Figure 2 represents the example of multiplex PCR for protocol I. Table 2 shows the deletion status detected using protocols I, II, and III. Using protocol I, a total of 41 infertile men were found to have Y deletions, yielding a deletion frequency of 6.5%. In contrast, no deletions were detected in the control group of 212 fertile men. There were four different deletion patterns observed using protocol I: *DAZ* deletion alone (30 patients), *DAZ, SMCY*, and *RBM1* deletions (8 patients), *USP9Y* deletion alone (1 patient), and *DBY* deletion alone (2 patients).

**FIGURE 2**

Representative multiplex PCR analysis of protocol I. (**Multiplex A**) Lane 1 = marker, lane 2 = DNA of a fertile man, lane 3 = DNA of a woman, lane 4 = DNA of a patient with *DAZ* deletion, lane 5 = DNA of a patient with *SMCY, RBM1*, and *DAZ* deletion, lane 6 = water control. (**Multiplex B**) Lane 1 = marker, lane 2 = DNA of a fertile man, lane 3 = DNA of a woman, lane 4 = DNA of a patient with *DBY* deletion, lane 5 = DNA of a patient with *USP9Y* deletion, lane 6 = water control.

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### Deletions Detected Using Protocol II

Using the 14-marker screen (protocol II), 41 infertile patients were found to have Y deletions, and no deletions were detected in the control group. The 41 patients identified by protocol II were the same as those identified by protocol I, suggesting that PCR protocol I could detect the same Y deletion cases identified by PCR protocol II. Using protocol II, five different deletion patterns were observed. One patient was found to have an isolated *USP9Y* deletion. Two patients were found to have isolated *DBY* deletions. These three patients with AZFa deletions were the same as those identified by protocol I. Thirty-eight patients had deletions encompassing multiple genes. All patients with the *DAZ* deletion alone, as detected by protocol I, were found to have *DAZ, CDY1*, and *BPY2* deleted (30 patients), as detected by protocol II. Patients with *DAZ, SMCY*, and *RBM1* gene deletions, as detected by protocol I, were found to have *CDY2, XKRY, SMCY, EIF1AY, RBM1, DAZ*, and *BPY2*

**TABLE 2**

**Deletion status of Y-chromosomal AZFs.**

| Patient | Age (y) | Semen analysis ($\times 10^6$/mL) | Testicular histology | Protocol I | Protocol II | Protocol III |
|---|---|---|---|---|---|---|
| 1 | 30 | 0 | SCOS | *USP9Y* | *USP9Y* | No deletion |
| 2 | 35 | 0 | SCOS | *DBY* | *DBY* | No deletion |
| 3 | 29 | 1.3 | NA | *DBY* | *DBY* | No deletion |
| 4 | 30 | 0 | MA | *SMCY, RBM1, DAZ* | *SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 5 | 31 | 0 | MA | *SMCY, RBM1, DAZ* | *SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 6 | 34 | 0 | HS | *SMCY, RBM1, DAZ* | *SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 7 | 28 | 0 | NA | *SMCY, RBM1, DAZ* | *SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 8 | 32 | 0 | MA | *SMCY, RBM1, DAZ* | *SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 9 | 36 | 0 | NA | *SMCY, RBM1, DAZ* | *SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 10 | 36 | 0 | HS | *SMCY, RBM1, DAZ* | *CDY2, XKRY, SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 11 | 31 | 0 | SCOS | *SMCY, RBM1, DAZ* | *CDY2, XKRY, SMCY, EIF1AY, RBM1, DAZ, BPY2* | *sY127, sY134, sY254, sY255* |
| 12 | 31 | 2.0 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 13 | 39 | 1.6 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 14 | 24 | <0.1 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 15 | 35 | 0 | HS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 16 | 31 | 8.8 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 17 | 27 | 0 | HS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 18 | 26 | 0 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 19 | 33 | 0 | MA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 20 | 34 | 7.9 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 21 | 33 | 0 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 22 | 43 | 0.7 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 23 | 28 | 0 | HS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 24 | 33 | 0 | SCOS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 25 | 28 | 0 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 26 | 34 | 1.9 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 27 | 39 | <0.1 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 28 | 41 | 0 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 29 | 36 | 0 | MA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 30 | 36 | <0.1 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 31 | 32 | 0.8 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 32 | 35 | 0 | HS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 33 | 25 | 0.3 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |

*Teng. Gene screen for Y chromosome deletions. Fertil Steril 2007.*

**TABLE 2**

**Continued.**

| Patient | Age (y) | Semen analysis ($\times 10^6$/mL) | Testicular histology | Protocol I | Protocol II | Protocol III |
|---|---|---|---|---|---|---|
| 34 | 34 | <0.1 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 35 | 36 | 0 | HS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 36 | 27 | 0.6 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 37 | 32 | 0 | HS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 38 | 29 | 0 | HS | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 39 | 30 | 0 | MA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 40 | 28 | <0.1 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |
| 41 | 36 | 2 | NA | *DAZ* | *DAZ, BPY2, CDY1* | *sY254, sY255* |

*Note:* HS = hypospermatogenesis; MA = maturation arrest; SCOS = Sertoli cell-only syndrome; NA = not available.

*Teng. Gene screen for Y chromosome deletions. Fertil Steril 2007.*

genes deleted (2 patients) or *SMCY, EIF1AY, RBM1, DAZ*, and *BPY2* genes deleted (6 patients), as detected by protocol II (Table 2).

### Deletions Detected Using Protocol III

Using protocol III, 38 patients were found to have AZFb or AZFc deletions, and no deletions were detected in the control group. Two different deletion patterns were observed. Eight patients were found to have *sY127, sY134, sY254*, and *sY255* deletions (AZFb and AZFc), and 30 patients were found to have *sY254* and *sY255* deletions (AZFc). These 38 patients identified by protocol III were also identified by protocols I and II (patients 4–41 in Table 2). However, by using protocol III, no patients were found to have *Y84* or *sY86* deletion (AZFa) Therefore, patients 1–3, who carried isolated AZFa gene deletions, as detected by protocols I and II, were found to be "no deletion," as detected by protocol III.

### Validation of AZFa Gene Deletion

We further tested the seven STSs (*sY82, sY83, sY86, sY85, sY84, sY87*, and *sY88*) and two pairs of gene-specific primers (*USP9Y-1* and *DBY-1*) in three patients with isolated AZFa gene deletions. Figure 3 shows that these three patients lacked either *USP9Y/USP9Y-1* or *DBY/BY-1*, but possessed all flanking STSs.

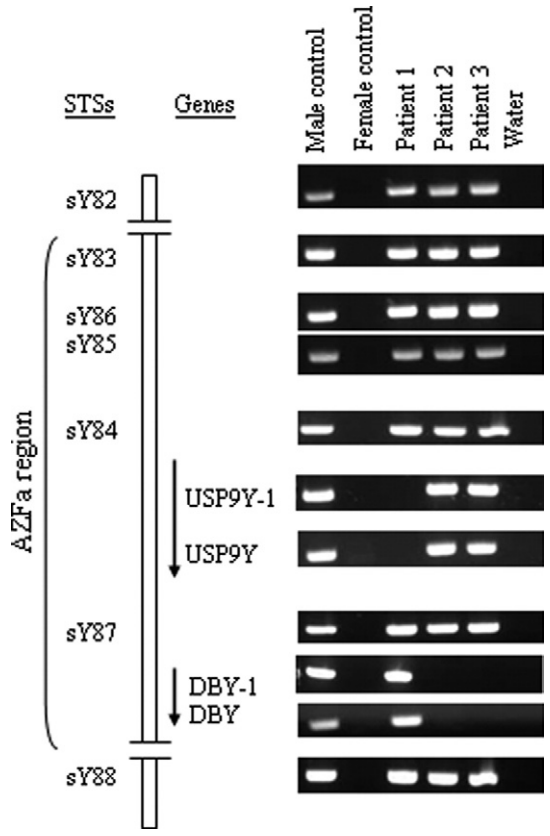
## DISCUSSION

The overall deletion frequency in this series was 6.5% (41/627). The deletion rate was 11.6% (34/293) in patients with sperm concentration $<1 \times 10^6$/mL or azoospermia, 4.5% (5/110) in patients with sperm concentration between 1 $\times 10^6$/mL and 5 $\times 10^6$/mL, and 0.9% (2/224) in patients with sperm concentration $>5 \times 10^6$/mL. Our findings are consistent with previous reports that Y deletions are found almost exclusively in patients with sperm concentration $<1 \times 10^6$/mL and are extremely rare with a sperm concentration $>5 \times 10^6$/mL (22, 23). In this study, two Y deletion patients (patients 16 and 20) with sperm concentrations of 8.8 $\times 10^6$/mL and 7.9 $\times 10^6$/mL raise the question of whether the Y deletion test should be routinely performed in patients with sperm concentration $>5 \times 10^6$/mL. Given the low deletion rate (0.9%) in this series and in other report (~0.7%) (24), it is reasonable to set the threshold for Y deletion testing at $<5 \times 10^6$/mL or nonobstructive azoospermia.

In the present study, a total of 41 patients were found to have Y deletions. These patients included three (7%) with AZFa, eight (20%) with AZFb + AZFc, and 30 (73%) with AZFc deletions. The observed deletion patterns and frequency are similar to those reported in United States (25), Italy (3), Germany (14), and India (26). Moreover, a review of more than 4,800 infertile patients showed that Y deletions most frequently involved the AZFc region (60%), less frequently the AZFb region (25%), and in only 5% of cases the AZFa interval (23). Therefore, the worldwide incidence of Y

**FIGURE 3**

Validation of AZFa gene deletions. Results of testing DNAs from patients 1–3, a male control, and a female control are shown.




*Teng. Gene screen for Y chromosome deletions. Fertil Steril 2007.*

deletion is likely to be similar and no ethnic difference exists between Taiwanese Han and the white population.

The human Y chromosome contains many amplicons that are non-X transposed, non-X degenerated, repetitive sequences with nearly complete identity in male-specific regions. Homologous recombination between amplicons has been shown to generate deletions with resultant spermatogenic failure. This phenomenon explains why most Y-deleted patients have massive deletions encompassing multiple genes (15, 16, 27). There are several types of "large deletions" in the Y chromosome (Fig. 1). The AZFc region is composed completely of amplicons, and is particularly susceptible to deletions. Homologous recombination between amplicons b2 and b4 is probably the most common genetic cause of spermatogenic failure (16). The b2/b4 deletion spans 3.5 Mb and eliminates almost the entire AZFc region, including *BPY2, DAZ, CDY1, GSPG4LY, GALGA2LY, TTY3*, and *TTY4* (15, 16). In our previous work, we have identified a group of Taiwanese infertile men with a uniform, recurrent, AZFc deletion junction. The proximal breakpoints are clustered between *sY1197* and *sY1192*, and the distal breakpoints are clustered between *sY1054* and *sY1125* (28). These breakpoints are identical to those described by Kuroda-Kawaguchi et al. (16). In light of the uniform deletions observed, it is most likely that all AZFc-deleted patients in this study had *GSPG4LY, GALGA2LY, TTY3*, and *TTY4* genes deleted simultaneously. It is noteworthy that partial AZF deletions have been reported to account for male infertility. Several types of partial deletions involved in AZFc region have been proposed and designed as gr/gr, rg/gr, b2/b3, and b1/b3 deletions (29–34). The nonallelic homologous recombination-based mechanism causing intrachromosomal recombination and subsequent deletion has been identified as their major cause (34). The possible effects and the pathophysiological roles of these partial AZFc deletions are yet to be determined and at least some of them, for example, gr/gr deletion, appear to be associated with reduced sperm counts (29, 33, 35, 36). However, we are unable to detect the partial AZFc deletions using PCR markers of the present study.

Repping et al. (18) have identified three types of deletions involving the AZFb region. "Pure" AZFb deletions, in fact, extend from palindrome P5 to the proximal arm of palindrome P1 and encompass the proximal AZFc region, thereby removing 32 genes and transcripts, including *CDY2, XKRY, HSFY, CYorf15A, CYorf15B, SMCY, EIF1AY, RPS4Y2, RBM1, PRY,* and transcription units of some *TTY* genes. The second type of AZFb deletion extends from P5 to distal P1 and removes 42 genes and transcripts, while sparing the distal AZFc region. The third type of AZFb deletion extends from P4 to the distal arm of P1, and is similar to proximal P5/distal P1 in deletion size except that the proximal AZFb region is not removed, thus sparing the *CDY2, XKRY*, and *HSFY* genes. Furthermore, Ferlin et al. (37) identified a fourth type of AZFb deletion with proximal breakpoints located between *sY108* and *sY113* and distal breakpoints located between *sY129* and *sY134* in infertile men with severe spermatogenic defects (i.e., a partial AZFb deletion). In this study, we have identified eight patients with deletions involving the AZFb region. Of the eight patients, six had *SMCY, EIF1AY, RBM1, DAZ*, and *BPY2* gene deletions and sparing *XKRY* and *CDY2* genes, suggesting that these six patients may have P4/distal P1 deletions. Two patients had *XKRY, CDY2 SMCY, EIF1AY, RBM1, DAZ*, and *BPY2* gene deletions, suggesting that these two patients may have P5/distal P1 deletion. Nevertheless, the deletion models proposed by Repping et al. (18) were based on only a few patients. It would be worthwhile to map deletion junctions of our patients with AZFb + AZFc deletions.

From the literature, AZFa deletion has been shown to be rare, and is believed to be the result of recombination between homologous retroviral sequence blocks HERV15yq1 and HERV15yq2 (38–40). This "complete" AZFa deletion will remove 792 kb including *sY83, sY84, sY85, sY86, sY87, USP9Y*, and *DBY* genes. Given that *sY84* and *sY86* are not polymorphic and one or both of their deletions could cause invariable testicular phenotype, that is, Sertoli cell-only syn-

drome (17, 24, 41–43), they have been recommended as representative STSs for the detection of AZFa deletions (14).

In the present study, one major discrepancy appeared between protocol I and protocol III. Three patients (patients 1–3) carried isolated *USP9Y* or *DBY* deletions detected by protocol I, and none of them could be identified by using protocol III. This discrepancy may be explained by the fact that Y chromosome is rich of repetitive sequences, which may undergo homologous recombination. It is possible that different deletion patterns other than complete AZFa deletion may exist. Thus, the isolated *USP9Y* or *DBY* deletions might not be detected by using *sY84* and *sY86*, which are located upstream of *USP9Y* and *DBY*.

From the literature, few cases have been described with partial AZFa deletions and variable testicular histologies (44–46). Patients with isolated *DBY* deletion would have testicular phenotypes of severe hypospermatogenesis or Sertoli cell-only syndrome, whereas the loss of *USP9Y* might be associated with severe hypospermatogenesis (45). One oligospermic man has been found to encompass *sY85* and *sY86* deletions but possess all flanking STSs of the AZFa region (46). In addition, a point mutation in *USP9Y* has been identified in one man presenting with spermatogenic arrest at the spermatid stage (47). In the present study, we designed additional experiments to validate the partial AZFa deletions: at least two more amplification failures by single PCR, amplification failures by the other set of gene-specific primers (*USP9Y-1* and *DBY-1*), and using a series set of STSs for the determination of the extensions of the deletions. Moreover, we confirmed that all four pairs of primer for *USP9Y* and *DBY* are not located in the polymorphic sites of both genes (48). Therefore, we tend to believe that the partial AZFa deletions are present in Taiwanese Han population. However, the obtained results were not fully confirmed. Given the extreme rarity of isolated AZFa gene deletion, the results obtained by PCR should be further confirmed by Southern blotting or sequence analysis. However, we were unable to perform these two experiments in these three patients because of insufficient DNA. Moreover, we could not confirm that the isolated *USP9Y* or *DBY* deletion is a de-novo event, because none of these three patients' male relatives agreed to provide blood sample for testing.

There is still considerable heterogeneity in PCR protocols for Y deletion test. Although some individual laboratories have developed their own protocols, the EAA/EMQN protocol is widely accepted and is supported by the American Urological Association, American Association of Bioanalysis, American Society for Reproductive Medicine, Human Fertilisation and Embryo Authority, and French Society for Human Genetics. In addition, some commercial kits are also available, for example, Promega Y Chromosome Deletion Detection System 2.0 kit (PROMEGA Corp., Madision, WI) and Bird-Set Y chromosome UE kit and Y chromosome Extension kit (BIRD srl, Caronno Pertusella, Italy) (49). Although various protocols have been designed, most of them used *sY84* and *sY86* markers for the detection of AZFa deletions. Given that isolated *USP9Y* or *DBY* deletions have been detected in our patients and in other groups, it is reasonable to suggest that the primers used for AZFa region should include not only *sY84* and *sY86* but also *USP9Y* and *DBY*. Once a partial AZFa deletion is found, it is important to define the deletion breakpoint to discover new deletion patterns.

At present, there is still debate about STS primers or gene-specific primers used to screen Y chromosome deletions. In most studies, a varied number of selected STSs were used, most of which are anonymous markers. Screening with anonymous STS markers may help in identifying regions where potential candidate genes or regulatory elements are located. In contrast, gene-specific screening provides a rational tool for genotype–phenotype correlation, which may contribute more information about the pathological role of the genes deleted. Although it has been shown that gene-specific screening did not give any advantage in the clinical management of the patients (41–43), our study showed that gene-specific screening (five markers) at least provides the same diagnostic efficiency as the EAA/EMQN protocol, and could be considered an alternative to the widely accepted EAA/EMQN protocol.

In summary, we observe consistent results between our PCR protocols and EAA/EMQN protocol studies, except for the three patients with isolated AZFa gene deletions. Although further studies are needed to confirm their existence, we believe that the protocol consisting of five markers (*USP9Y, DBY, SMCY, RBM1*, and *DAZ*) would constitute a rational first-line screening panel. This gene-specific protocol will significantly reduce the workload while attaining the same diagnostic efficiency as a more complex protocol. After the first-line screening, a more sophisticated protocol would be required to identify rare cases with novel deletion junctions or to determine the extensions of deletions.

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