A novel y chromosome microdeletion with the loss of an endogenous retrovirus related, testis specific transcript in AZFb region

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Title

A novel Y chromosome microdeletion with the loss of an endogenous retrovirus (ERV)-related testis-specific transcript in AZFb region

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key words

male infertility, Y chromosome, AZF, homologous recombination, ERV

Abstract

Purpose: This study was designed to identify the endogenous retroviruses (ERVs) associated with testis-specific transcripts linked to the Y (TTYs) in the azoospermia factor (AZF)b region. We evaluated the relationship between ERVs, TTY expression patterns, and TTY function in spermatogenesis.

Material and Methods: Identification of TTY family members in the AZFb region was performed using computational screening. After investigating the relationship between ERV genome and TTY expression patterns. We screened genomic PCR products from *TTY13* amplified from 790 individuals: 275 azoospermia patients, 285 oligozoospermia patients, and 230 fertile males in Japanese subjects.

Results: Computational screening revealed three TTY family members (*TTY9*, *10,* and *13*) regulated by ERVs in the AZFb region. Homologous recombination between LTR of a *TTY13*-associated ERV, HERV-K14C, resulted in *TTY13* deletion events. These deletions were more frequent in azoospermic and oligozoospermic patients than in fertile males. Specifically 15.63% of the azoospermia group had only the deletion variant, 10.88% of the oligozoospermia group, and 0% of the fertile controls indicating that there is an association between the rate of homologous recombination and the severity of spermatogenesis failure that shows statistical significances ($p<0.05$).

Conclusions

The finding of novel micro-deletions due to ERV in the AZFb indicated that our study raises the possibility that specific variations in genomic structure may contribute to some forms of human idiopathic male infertility.

Introduction

Approximately 8% of the human genome is composed of ERVs and related sequences [1]. It is generally believed that exogenous retrovirus infected ancient host germ-cells and formed proviruses in their genomes. The majority of ERV families integrated into the primate genome after the divergence of New World and Old World monkeys, and subsequently amplified several times during primate evolution [2]. ERVs contribute to the host genome and can be associated with the pathogenesis of autoimmune disease, psychiatric disease, cancers, and male infertility [3, 4]. Retroviral genomes are flanked by LTRs that encode regulatory elements that potentially provide enhancers, alternative promoters, and polyadenylational signals to nearby cellular genes [5]. Several studies report that EVR LTR elements function as promoters and enhancers of gene expression in specific tissues and cell lines [3].

All Y-chromosome sequences can be classified as X-transposed, X-degenerate, or ampliconic. The ampliconic regions comprise eight palindromes that share more than 99.9% sequence homology [6]. Microdeletions on the long arm of the Y chromosome contribute to male infertility. The AZF region is thought to be rich in various functional genes and transcription units, and is essential for spermatogenesis [7]. Although the AZF region is divided into three regions, AZFa, AZFb, and AZFc, according to chromosomal position and testicular pathology, part of AZFb region is reported to overlap with part of AZFc [8]. Many such microdeletions identified to date result from non-reciprocal

65 intrachromosomal recombination events between homologous sequences and lead to
66 genome variation and rearrangement [9].
67 Deletions of AZFa can result from non-reciprocal homologous recombination events
68 between two HERV sequence elements [6, 10]. The AZFb region consists of long tracks of
69 repeated sequences. Genes reside in this interval, and most of them encode testis-
70 specific transcripts [8, 11]. The AZFc region is composed entirely of amplicons, and it is
71 particularly susceptible to deletion. The palindromes in the AZFc region consist of a
72 complex of several small segments called sub-amplicons [12]. Deletions in the AZFc
73 region were shown to result from recombination between two direct repeats, sub-
74 amplicon b2 and b4 [12]. Therefore, microdeletions have removed several testis-specific
75 transcription and expression units, and reduction in the copy number of AZFc genes
76 could cause reduced sperm production [6, 8, 13, 14].
77 Transcripts of the TTY family are dispersed throughout ampliconic and X-degenerated
78 regions of the Y chromosome; however, their role in spermatogenesis, predicted by *in*
79 *silico* analysis, is still poorly understood. Although the TTY family was thought to be
80 expressed exclusively in testis, recent studies have shown some TTYs are not always
81 testis-specific [8, 15].
82 This study was designed to identify the ERVs associated with TTYs in the AZFb region
83 using computational screening. We sought to understand the relationship between ERVs,
84 TTY expression patterns, and TTY function in spermatogenesis in Japanese subjects.

Materials and Methods

Study population

The Ethics Committee of Kanazawa University Hospital approved the study and informed consent was obtained from all participants. From April 2006 to March 2009, we recruited patients with azoospermic and oligozoospermic ejaculate. We excluded individuals with abnormal karyotypes and Y-chromosomal microdeletions. Some patients with azoospermia underwent open testicular biopsy or retrieval of sperm from testicular tissues for histological evaluation and/or TESE in a pilot study. Normal testicular samples were obtained from obstructive azoospermic patients who had undergone a diagnostic testicular biopsy. After this pilot study, we performed the genotype analysis of fertile males (controls) and patients with azoospermia or oligozoospermia. Our control group comprised 230 male volunteers, all of whom were healthy young men who had fathered at least one healthy child without any assisted reproductive procedures.

Histological evaluation and selected subjects in the pilot study

To diagnose the spermatogenic pattern, samples were obtained from at least three different areas in the testis. Testicular histology was classified into four categories: hypospermatogenesis, MA, SCO, and tubular sclerosis.

Thirty-three subjects were selected and grouped; Tubular sclerosis (a, no. 1-5) and SCO subjects (b, no. 6-10) represent histologically no cells in the tubular section and no germ

cells but SCO in the whole seminiferous tubule, respectively. MA (c, no.11-20) represent no spermatids or sperms, despite much mitotic activity. Hypospermatogenesis subjects (d, no.21-30) represent the reduction in the number of germ cells at all stages. All affected cases show azoospermia in the ejaculate. Normal spermatogenesis (d, no.31-33) shows normal appearance histologically.

Mapping and computational screening for ERV-related TTYs

GenBank data for the human Y chromosome in contig NT_011875 was used in this study. RefSeq mRNAs and genomic loci were identified by the RepeatMasker program (http://repeatmasker.genome.washington.edu) with various repeat element consensus sequences from the Repbase Update [16]. After analyzing RefSeq mRNA sequences, we reconstructed the genomic structure of ERV-related TTY family members using PipMaker (http://pipmaker.bx.psu.edu).

Preparation of genomic DNA and RNA samples

Testis tissue RNAs were isolated from infertile TESE patients using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA from male human tissues and from human ovary was purchased from Clontech (Mountain View, CA, USA). Pure mRNA was obtained using the PolyA Tract mRNA isolation system (Promega, Madison, WI, USA). All genomic DNA was isolated from peripheral blood samples with a genomic-tip kit (Qiagen, Hilden, Germany).

RT-PCR amplification

cDNA samples from human total RNA were synthesized with MMLV-derived reverse transcriptase with oligo (dT) and random hexamer primers (Promega). Genomic PCR and RT-PCR reactions were carried out with LA Taq Hot Version kit and a standard PCR kit supplied by Takara (Kyoto, Japan). Primer information and accession numbers are documented in supplementary Table (http://web.kanazawa-u.ac.jp/~med29/andrology/supplementary.html), and primer positions are shown Fig. 1C,D, and E. As a positive control, *GAPDH* was amplified.

Sequencing of deletion junctions

PCR products were sequenced on an ABI 337 DNA sequencer. The same primers that had been used for amplification were used, along with the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, City, CA, USA) for the sequencing reactions.

Genomic DNA and semen examination

All blood samples were derived from Japanese subjects. The control group comprised 230 confirmed fertile male volunteers. All infertile patients had normal karyotypes. Deletions of AZF region were performed as described previously[14]. These infertile patients were classified as having azoospermia or oligozoospermia ($<20 \times 10^6\ ml^{-1}$) in the ejaculate. By these criteria, 275 patients had azoospermia, and 285 patients had oligozoospermia.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences statistical software version 11.0 (SPSS, Chicago, IL, USA). The Mann-Whitney test was used to determine whether there was any significant difference in genotype frequencies between the normal control and infertile male groups. The value of $P < 0.05$ was taken to be statistically significant.

Results

Genomic structure and position of three ERV-related TTY family members

We screened the Y-chromosome genomic contig (NT_011875) from GenBank with the RepeatMasker program and identified three candidate ERV-related sequences: *TTY9*, *TTY 10*, and *TTY 13*, and constructed genomic structures using PipMaker. *TTY9*, *TTY 10*, and *TTY 13* were dispersed throughout the AZFb region between P4 and IR2 (Fig. 1B). On the basis of computational analysis, we determined the structure and Y-chromosome location of the three ERV-derived transcripts (Fig. 1B).

Two copies of *TTY9*, *TTY9A* and *TTY9B*, are identical (Fig. 1B); this identity most likely results from the duplication of P4. Internal sequences of ERV3 provided the first exon and the promoter region for *TTY9* (Fig. 1C). Using TRANSFAC® Professional version 10.1 with a strict threshold (core match: 1 and matrix match: 0.95~1), we found six binding sites for multiple transcription factors in the promoter region distributed upstream of the transcription starting site.

TTY10 contained two types of ERV-related sequences, LTR12B and LTR19C. LTR12B provided the 3rd and 4th exons of *TTY10*, and LTR19C contributed to the 5th and 6th exons of *TTY10* (Fig. 1D). The 4th and 5th exons encoded the ORF indicating that both ERVs, LTR12B and LTR19C, harbored exons and functional coding regions.

TTY13 contained HERV-K14C, including both LTRs and partially deleted internal sequences (5'LTR-gag-pro-env-3'LTR, and an *AluYd* insert), spanned the last two exons of *TTY13*. HERV-K14C provided the 5th exon, which contained an ORF, and the 6th exon (Fig. 1E).

Expression patterns of the ERV-related TTY family

We used RT-PCR to examine the expression profiles of TTYs in various human tissues. The TTY family is thought to be exclusively or predominantly expressed in the testis, consistent with their location on the Y chromosome. RT-PCR analysis revealed that *TTY9* was expressed in testis and kidney and that *TTY10* was expressed in all tissues examined except the ovary (Fig. 2). In contrast, *TTY13* expression was testis-specific. Although *TTY9* and *10* transcripts were observed in other tissues, the transcripts seemed to be most abundant in testis. A number of genes and transcripts are dispersed throughout AZFb on the MSY, and individual deletions of these sequences are thought to cause failure of spermatogenesis. Therefore, we suggest that the ERV-related TTYs participate in spermatogenesis. To investigate the relationship between ERV-related TTYs and spermatogenesis, we analyzed the genomic DNA that encodes TTYs and

testis-specific RNAs from patients that present with different types of spermatogenic failure and the same regions of genomic DNA from normal testes.

PCR analysis of genomic DNA in the ERV-related TTY family

We amplified the genomic regions flanking each ERV-related TTY to avoid amplifying other ERV-related sequences in the genome (Fig. 3A). The expected size of the PCR product with the ERV associated with *TTY13*, HERV-K14C, was 6073 nucleotides, and PCR reactions identified three genotypes; intact 6,703-bp product, a smaller 668-bp product, and both products (Fig. 3A). Both the 6,703-bp product and a smaller 668-bp product were amplified from most patients, but seven individuals (1, 7, 14, 15, 22, 26, and 33) presented with only the intact product. In addition, some samples (patients 3, 13, and 27) only yielded only the smaller 668-bp product.

To confirm of this finding, we applied other STS marker to all subjects. The primers were designed that one was located in deletion discrepancy (primer pair; S5, AS5) and the other was resided in flanking region of *TTY13* locus ((primer pair; S6, AS6). We estimated that PCR product was not detected in our subjects when deletion was occurred. As depicted in Fig 3A, three individuals who showed smaller 668-bp product which were not revealed any other bands in their genomic DNAs.

RT-PCR analysis of testicular RNA in the ERV-related TTY family

We also analyzed testis RNA from individual infertile patients and normal controls using RT-PCR. No significant relationships between expression of the ERV-related

TTY families and idiopathic infertility were detected (Fig. 3B). We found that the ERV-related TTY family was transcribed from testes with tubular sclerosis and those with no germ cells (Fig. 3B a and b) suggesting these TTYs may play a role in the somatic cells of testis tissues.

However, in three samples (3, 13, and 27) from three separate histological categories (tubular sclerosis, MA, and hypospermatogenesis) intact *TTY13* was not transcribed; this RT-PCR analysis was consistent with the genomic DNA analysis in that there was only one smaller transcript in these individuals.

TTY13 deletion junction indicates homologous recombination

In our pilot study, the smaller *TTY13* PCR product seen in patients 3, 13, and 27 indicates that these individuals harbor a deletion in this region of their genome. To test this hypothesis, we sequenced the PCR amplified DNA from this genomic region. As shown in Fig. 4, the sequences amplified from these patients align with the 5' LTR and 3' LTR of HERV-K14C from the *TTY13* locus. This analysis also revealed a stretch of 149 overlapping sequences shared by the 5' LTR, the 3' LTR, and the genomic DNA, suggesting that this stretch is a homologous recombination junction point. This stretch of genomic DNA has an overall sequence identity of approximately 88% with the LTRs, and some regions of the junction point showed perfect matches with both LTRs (Fig. 4A). The homologous recombination event eliminated 5,405 nucleotides—including introns, the last two exons, and the *AluYd* insert—from the AZFb region (Fig. 4B). This

result suggested that *TTY13* was subject to the gain and subsequent loss of HERV-K14C sequences, resulting in an interruption of *TTY13* expression.

Frequency of homologous recombination *TTY13* variant in fertile and infertile men

Infertile individuals, specifically patients 3, 13, and 27, harbored the deletion variant of the *TTY13* locus. Therefore, to estimate the frequency of homologous recombination associated with idiopathic male infertility, we screened genomic PCR products from *TTY13* amplified from 790 individuals: 275 azoospermia patients, 285 oligozoospermia patients, and 230 fertile males (Table). The mean age of proven fertile control males was 31.2 ±4.5 years; the ages ranged from 20 to 43 years with a median of 31 years. The mean and median sperm concentrations were $105.6 \pm 78.8 \times 10^6\ mL^{-1}$ and $83.2 \times 10^6\ mL^{-1}$, respectively; the range of sperm concentrations was $2.2 \times 10^6\ mL^{-1}$ to $438 \times 10^6\ mL^{-1}$.

All the samples were tested with two of primer set for genomic DNA amplification to confirm the deletion event. The most common genotype, representing 82.4% of all individuals, had both deletion and intact (del/int) variants of HERV-K14C from the *TTY13* locus in genomic DNA.

Interestingly, individuals harboring only the deletion variant were restricted to infertile patients, and none were observed among the fertile controls. In addition, the percentage of individuals that carried only the deletion variant within a group was correlated with the severity of spermatogenic failure in that group. Specifically 15.63% of the

azoospermia group carried only the deletion variant, 10.88% of the oligozoospermia group, and 0% of the fertile controls (Table). These results indicate that there is an association between the rate of homologous recombination and the severity of spermatogenesis failure and that this association is statistically significantly ($p<0.05$). Additionally in the fertile control group, the genotype with only an intact *TTY13* locus occurs at a relatively high frequency.

Discussion

Many researchers who study male infertility have investigated deletions of ampliconic sequences in the AZFa, AZFb, and AZFc regions. Microdeletions in the AZFa regions are thought to result from homologous recombination events due to human ERVs [10, 17]. Some studies reporting on deletion breakpoints in P5/proximal-P1 (AZFb), P5/distal-P1, P4/distal P1 explained only those deletions resulting from direct repeat sequences[8].

Male infertility and several other human diseases result from recurrent DNA rearrangement due to homologous recombination involving unstable genomic regions [3]. Here, we report that ERVs influence the expression of *TTY9, TTY10,* and *TTY13* in the AZFb region and that the ERV in *TTY13,* HERV-K14C, mediates recurrent homologous recombination events. For example, ERV3 acts as promoter for *TTY9* (Fig. 1C). Moreover, ERV sequences were incorporated into 3' exons of *TTY10* and *TTY13* and are presumed to contribute to *TTY10* and *TTY13* transcriptional termination (Fig. 1D, E).

Unlike TTY9 and TTY10, both the intact and the deletion form of *TTY13* (6073 and 668 bp, respectively) are present. During mitosis or meiosis, sister chromatids exchange genetic information through homologous recombination via intrachromosomal rearrangement [18, 19]. In addition, a single chromatid fold-back lariat mediated by directly oriented repeats can lead to non-reciprocal crossing-over and cause excision of the lariat (Fig. 4). This intrachromatid homologous recombination can occur in both somatic and germ cells [20]. When it occurs in germ cells, the new genetic deletion variant may be directly transmitted to the progeny.

We observed both the intact and the deletion form of *TTY13* (6073 and 668 bp, respectively) in genomic DNA, indicating that recombination events are frequent and occur predominantly by the LTR mechanism we propose. In addition, only 65 (20 infertile and 45 fertile) males harbored only the intact HERV-K14C sequences, suggesting that neither the intact nor deleted form is fixed in the human population.

In particular, the HERV-K elements, members of the ERV family, have been continuously multiplying in the human genome, which has led to polymorphism in the human population [21, 22]. HERVs become inactivated over time, and inactivated HERVs can lead to solitary LTRs, which result from homologous recombination between the two LTRs flanking the provirus and the subsequent deletion of the internal sequence [23, 24]. Some genetic variation was due to the presence of HERV-K solitary LTRs instead of the full-length counterpart in a small number of human individuals. Homologous

recombination between the 5’ and 3’ LTRs of HERV-H resulted in the presence of intact and deleted variants in the same individual, suggesting that this genetic variation is due to an LTR-LTR excision event in humans [25].

We proposed that HERV-K14C mediates homologous recombination events on the human Y chromosome by a similar mechanism. Screening of 790 (275 azoospermia, 285 oligozoospermia, and 230 fertile) males demonstrated that a subpopulation of infertile males harbored only the deletion variant (n = 74), whereas 567 (219 azoospermia, 247 oligozoospermia, and 185 fertile) of 790 samples, representing three fertility classes (azoospermia, oligozoospermia, and fertile), contained both the intact (6,073 bp) and deletion (668 bp) forms of *TTY13* (Table 2). The deletion variant was more common in infertile males and seemed to occur more frequently when the symptoms were more severe.

Repeat sequences are dispersed at low densities in ampliconic regions of the Y chromosome. In contrast, ERV-related sequences were more common in ampliconic region than in human genomic sequence on average [26, 27]. The evolutionary dynamics of the Y chromosome are generally much faster than those of the autosomes and the X chromosome, owing to deletions and mutations [28, 29]. Our data suggest that ERVs may lead to genomic instability by inducing new insertions and by causing deletions via homologous recombination of intrinsic ERV sequences, particularly LTRs. These

deletion events may be associated with some cases of male infertility that are due to genomic instability.

Conclusion

The finding of novel micro-deletions due to ERV in AZFb indicated that our study raises the possibility that specific variations in genomic structure may contribute to some forms of human idiopathic male infertility. These observations emphasize the necessity of investigating genome structure and transcripts of Y chromosome in detail.

Competing interest

The authors declare that they have no competing interests.

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Fig. legends

Fig. 1 Schematic diagrams of a Y-chromosome overview. (A) Overview of the Y chromosome: gray-scale boxes indicate pseudoautosomal (black), euchromatic (white), and heterochromatin (light gray) sequences. (B) The enlarged schematic representation of a palindrome region: gray-scale boxes indicate X-degenerate (pale gray), heterochromatin (medium gray), and ampliconic (dark gray) regions, palindromes are illustrated by dark gray arrows (P1-8) and AZFa,b,c regions. The locations and directions of ERV-related TTYs in AZFb are indicated as black triangles. The genomic structures of the ERV-related (C) *TTY9*, (D) *TTY10*, and (E) *TTY13* are illustrated. Boxes represent exons, and ORF are in black. ERVs are depicted by large dark gray arrows. Splicing is represented by solid lines, and small black arrows indicate PCR primer locations.

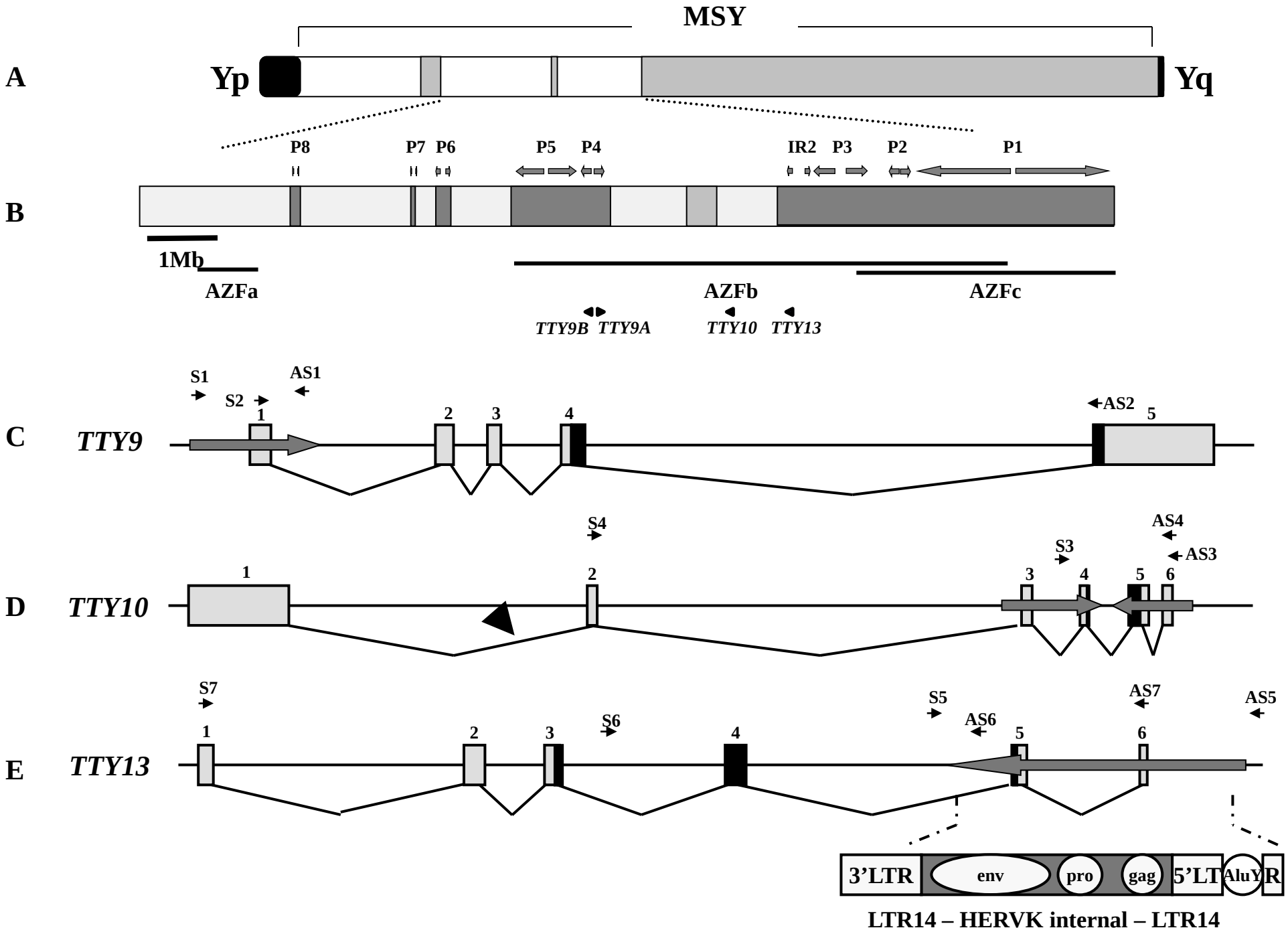
Fig. 2 Expression profiles of ERV-related TTYs. RT-PCR analysis of *TTY9*, *TTY10*, and *TTT13* expression in various human tissues. *GAPDH* was used as a positive control.

Fig. 3 PCR amplification of genomic DNAs and RT-PCR analysis of testis cDNA from azoospermic patients.

(A) PCR amplification of genomic DNA from peripheral blood cells, (B) RT-PCR of testis cDNA.

(A) and (B): M, size markers; 1-33, individual male subjects. The subjects are grouped as follows: a, Tubular sclerosis; b, SCO; c, MA; d, hypospermatogenesis; and e, normal spermatogenesis (Materials and Methods). *GAPDH* was used as a positive control.

Fig. 4 *TTY13* deletion junction indicating homologous recombination between 5' and 3' LTR of HERV-K14C. (A) Analysis of sequence data from the 668-bp deleted form, the 5' LTR, and the 3' LTR. The breakpoints between both LTRs are indicated with gray boxes. Open boxes illustrate the genomic region flanking HERV-K14C. (B) Schematic representation of the putative homologous recombination between the 5' and 3'LTRs. Gray pentagons depict recombination spots. The black box indicates an *AluYd* insert.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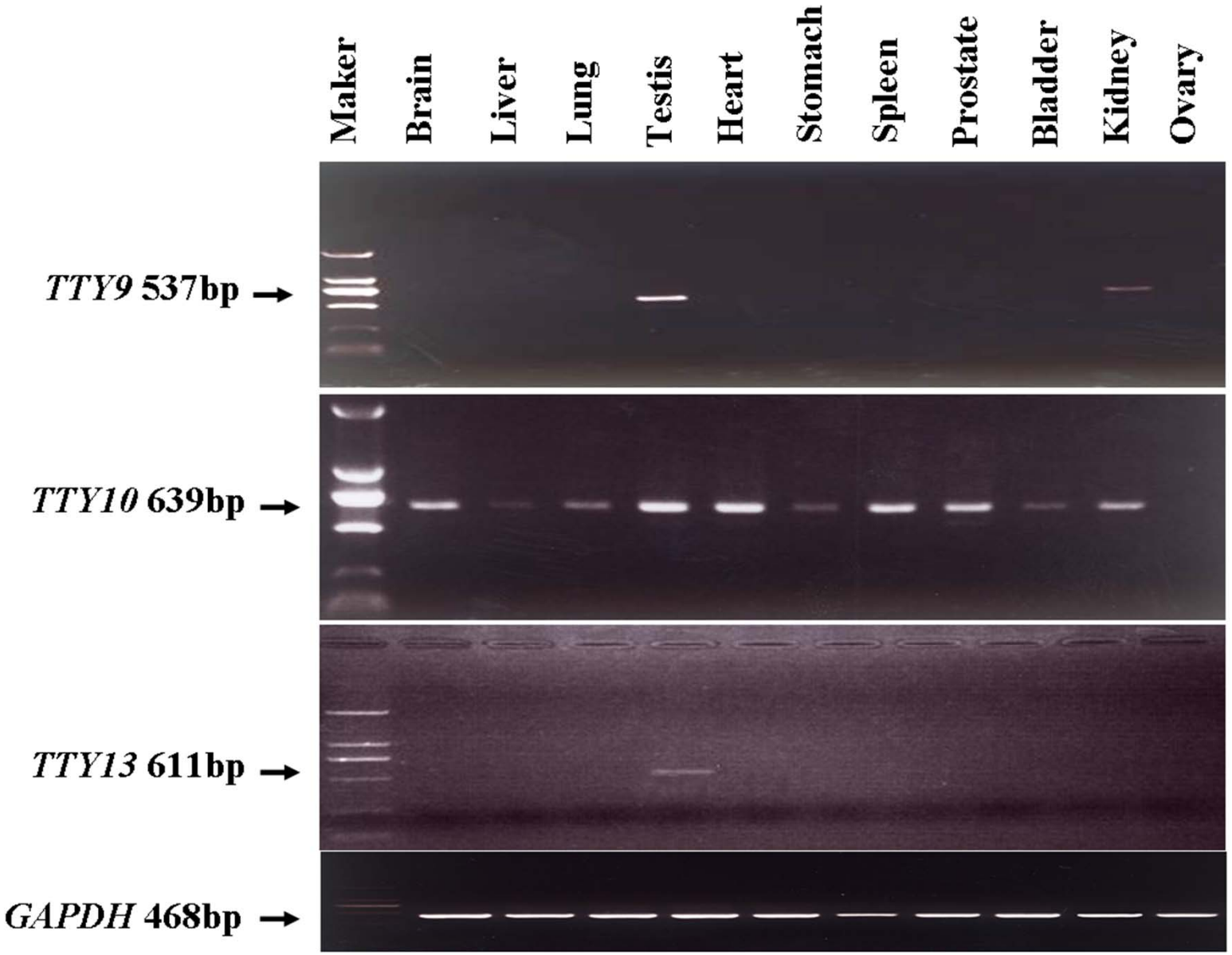
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Kidney
Ovary
TTY9 537bp
TTY10 639bp
TTY13 611bp
GAPDH 468bp

A

Maker a 1 2 3 4 5 b 6 7 8 9 10 Maker c 11 12 13 14 15 16 17 18 19 20 Maker d 21 22 23 24 25 26 27 28 29 30 Maker e 31 32 33

TTY9 **3667bp**
(probes; S1, AS1)

TTY10 **3228bp**
(probes; S3, AS3)

TTY13 **6073bp**
(probes; S5, AS5)

668bp

TTY13 **2848bp**
(probes; S6, AS6)

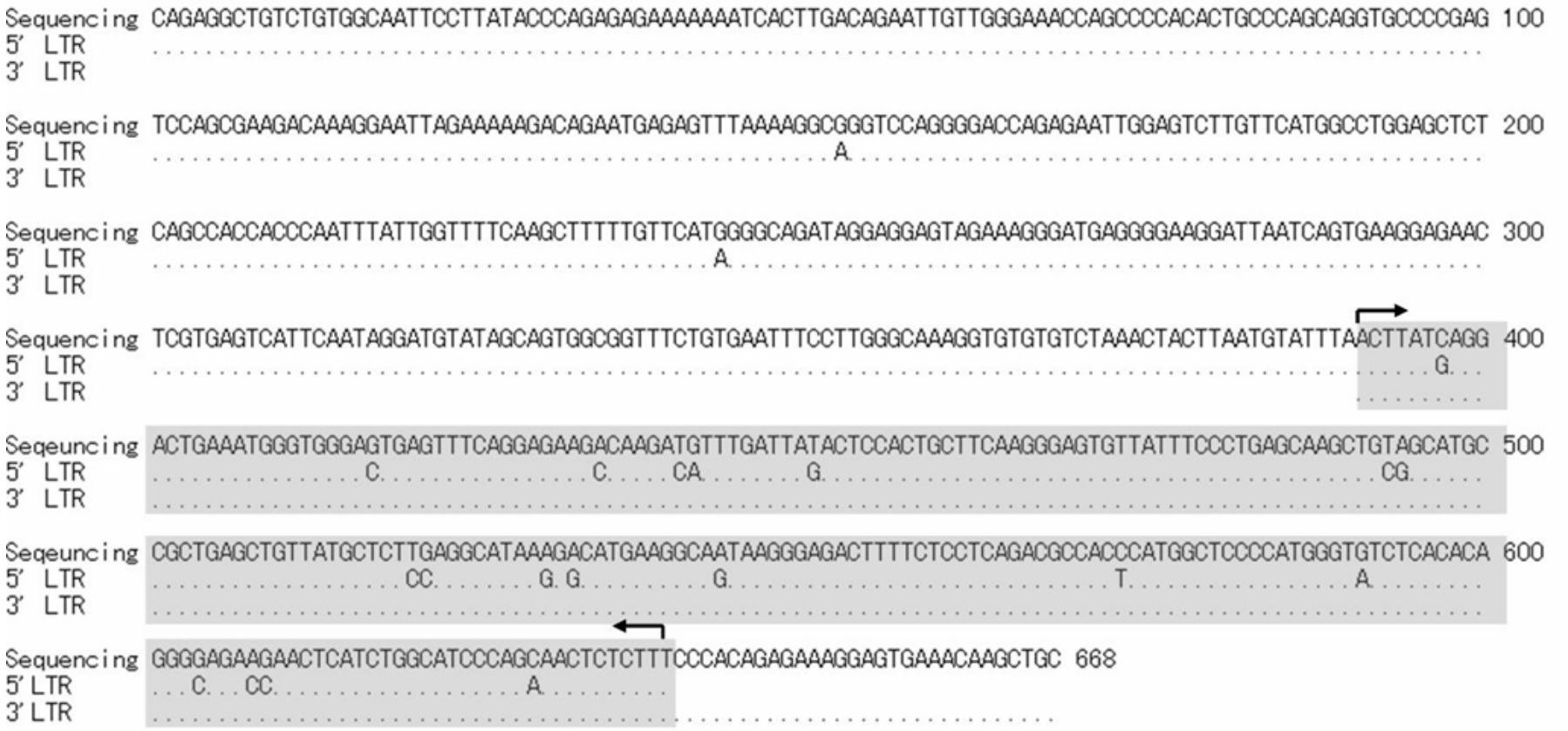
B

TTY9 **537bp**
(probes; S2. AS2)

TTY10 **639bp**
(probes; S4, AS4)

TTY13 **611bp**
(probes; S7, AS7)

GAPDH **468bp**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3′ LTR
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5′ LTR
3′ LTR
Sequencing CAGCCACCACCCAATTTATTGGTTTTCAAGCTTTTTGTTCATGGGGCAGATAGGAGGAGTAGAAAGGGATGAGGGGAAGGATTAATCAGTGAAGGAGAAC 300
5′ LTR
3′ LTR
Sequencing TCGTGAGTCATTCAATAGGATGTATAGCAGTGGCGGTTTCTGTGAATTTCCTTGGGCAAAGGTGTGTGTCTAAACTACTTAATGTATTTAACTTATCAGG 400
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3′ LTR
Seqeuncing ACTGAAATGGGTGGGAGTGAGTTTCAGGAGAAGACAAGATGTTTGATTATACTCCACTGCTTCAAGGGAGTGTTATTTCCCTGAGCAAGCTGTAGCATGC 500
5′ LTR
3′ LTR
Seqeuncing CGCTGAGCTGTTATGCTCTTGAGGCATAAAGACATGAAGGCAATAAGGGAGACTTTTCTCCTCAGACGCCACCCATGGCTCCCCATGGGTGTCTCACACA 600
5′ LTR
3′ LTR
Sequencing GGGGAGAAGAACTCATCTGGCATCCCAGCAACTCTCTTTCCCACAGAGAAAGGAGTGAAACAAGCTGC 668
5′LTR
3′LTR

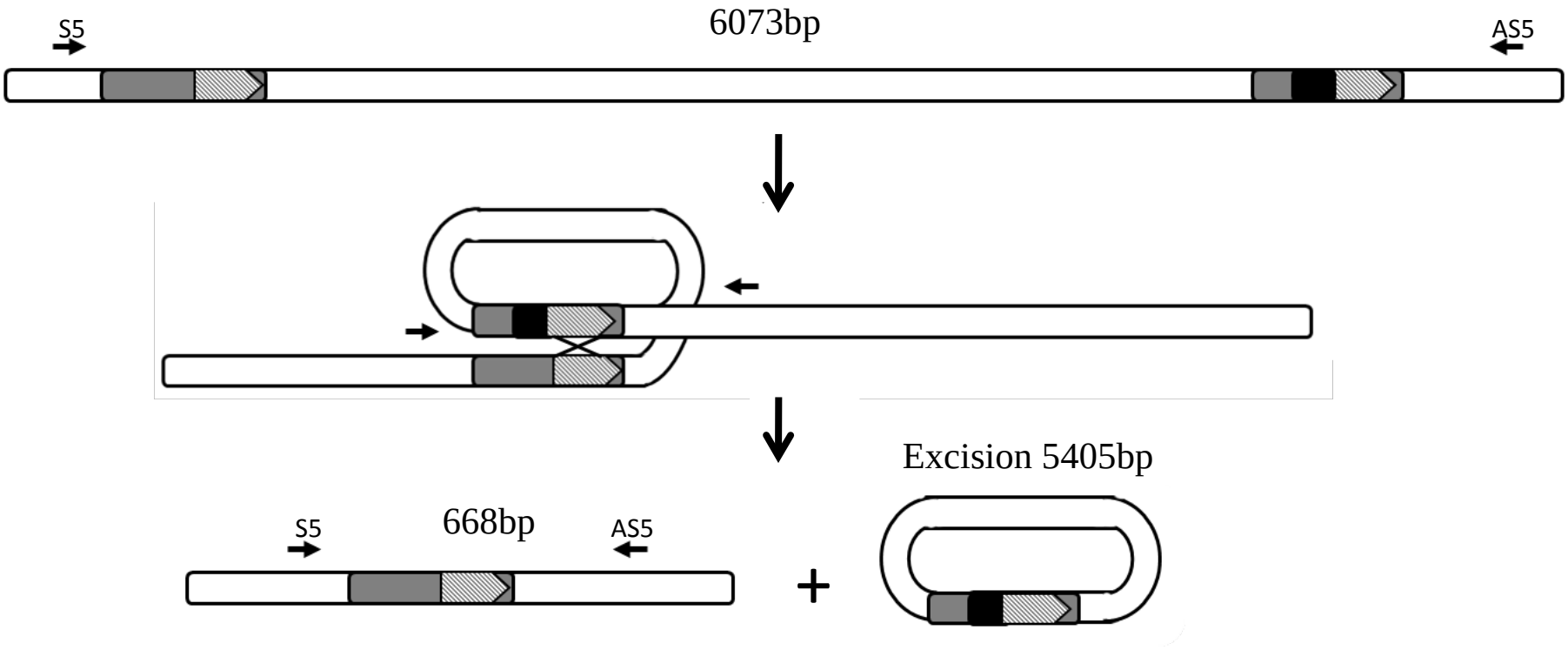
S5
6073bp
AS5
668bp
S5
AS5
Excision 5405bp

Table The frequency of TTY13 genotypes in infertile male patients and normal subjects

Variant types		number	intact	int/del	Deletion
Normal controls					
	Known fertility	n = 230	45 (19.6%)	185 (80.4%)	0 (0%)
Infertile patients					
	Oligozoospermia*	n = 285	7 (2.46%)	247 (86.66%)	31 (10.88%)
	Azoospermia*	n = 275	13 (4.72%)	219 (79.63%)	43 (15.63%)

int/del; intact+deletion variants

* Mann-Whitney U test ($p<0.05$) when compared with known fertility group.

Supplemental Table Primers for genomic and RT-PCR analysis

Gene	Primers	Sequence 5'- 3'	Size	RefSeq
TTY9	S1	GCTCAATCTCTGCCTACTGG	3677bp	NT_011875
	AS1	ACTCAAGCCAGGGTGACAGG		
	S2	CAACAGCCCTGCTCTGGTCC	537bp	NR_002159
	AS2	GCAAACCTGGTTACCAAGAG		
TTY10	S3	CATGTGAGAAGCCAGCACTGAC	3228bp	NT_011875
	AS3	CTTATTCCCTGATCAGGTAGGC		
	S4	CATTGGAGAATCAGGTCCAG	639bp	NR 001542
	AS4	CTTATTCCCTGATCAGGTAG		
TTY13	S5	CAGAGGCTGTCTGTGGCAATTC	6073bp	NT_011875
	AS5	GCAGCTTGTTTCACTCCTTTCTC		
	S6	CTGTTGTAGCTTTGGATTCTTCTA	2848bp	NT_011875
	AS6	TATTTATTTATTTATTTGCAGGT		
	S7	CAAGCAGAGCCAAACAGACA	611bp	NR_001537
	AS7	GACCACCAGTAATCTAATGGT		
GAPDH	GAPDH-S	GCCACATCGCTCAGACAC C	468bp	NM_002046
	GAPDH -AS	GCTGATGATCTTGAGGCTGT		

The positions of primers is in Fig. C, D and E