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PCR for rapid detection of nine loci of Y-chromosomal DNA including SRY sequences in Turner syndrome patients

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Abstract

Detection of Y-chromosomal sequences is crucial for certain cases of phenotypic females since prophylactic gonadectomy is recommended to prevent germ cell tumor development in the future. This cross-sectional study aimed to develop a simple, rapid, and accurate method for determination of 9 loci of Y-chromosomal DNA spanning Yp, centromere, and Yq, including *SRY* sequences by using 2 polymerase chain reaction (PCR) conditions. The first PCR condition was used for Assay A, which yielded pentaplex PCR products consisting of loci *ZFX/ZFY*, *SRY*, *AZFz*, *AZFb*, and *AZFc*. The second PCR condition was used for Assay B, which yielded PCR products consisting loci *PABY*, *AMGLXX/AMGLXY*, Y-centromere, and YD. DNA from 20 Turner syndrome (TS) patients with different chromosomal aberrations were assessed. Normal female DNA and normal male DNA were used as controls. The pentaplex PCR together with 4 other simultaneous PCR reactions showed 10 positive PCR products of 9 loci in normal male controls and 3 mosaic TS patients (45,X/46,XY and 45,X/46,X,+marker). Five positive PCR products representing the regions *PABY*, *ZFX/ZFY*, and *AMGLXX/AMGLXY* and *SRY* were identified in 1 of 9 TS (monosomy; 45,X) patients. All female controls, 8 TS patients with monosomy X, and 8 mosaic TS patients with only X chromosome aberrations showed positive results for loci *ZFX/ZFY* and *AMGLXX*. The developed PCR conditions were demonstrated to accurately and rapidly detect 9 Y-chromosomal fragments including SRY sequences in TS patients.

Keywords: PCR, Y chromosome, SRY, Turner syndrome

1. Introduction

The Y chromosome comprises approximately 1% of DNA content of diploid human genome [1]. It is essential to have a Y chromosome for an embryonal undifferentiated gonad to develop into a testis. Many genes, alone or together with some other genes, play roles in the differentiation processes. The *SRY* gene located on the short arm of the Y chromosome (Yp), first described in 1990, encodes a protein that acts as a transcription activator controlling other genes essential for gonadal differentiation. Abnormalities of the *SRY* gene, such as nucleotide substitutions, microdeletions, or other gross lesions, could result in many phenotypic abnormalities. The Y chromosome also plays many other important roles in human reproduction. For example, microdeletions of *AZFab-c* are associated with inability to produce sperm [2-3]. Many other genes have been described to span the whole length of the Y chromosome [4]. Repeated satellite DNA sequences are also identified in some regions of the Y chromosome (e.g., the heterochromatin region of the Yq). The clinical importance of these genes has been recognized and also reported elsewhere [4].

There are many women who carry the Y chromosome or Y-chromosome sequences such as Turner syndrome (TS) patients. And this uncommon gynecological condition presents with delayed puberty and/or primary amenorrhea. The karyotype of a typical TS patient is 45, X monosomy. However, various chromosomal aberrations are reported in 40-50% of mosaic TS patients, including presence of the whole Y chromosome or

cryptic Y-chromosome sequences. For Turner mosaicism, patients can achieve puberty and menarche, but later present with secondary amenorrhea or premature ovarian insufficiency. The karyotypes of TS patients in the latter group have been reported in many variations, such as 45,X/46,XX; 45,X/46,XY; 45,X/46,X,+mar; and, 45,X/46,i(X)(q10). The prognosis of a TS patient also varies by karyotype. The most important considerations are high risk of developing gonadoblastoma or other gonadal tumors, and virilization at puberty which may occur if the Y chromosome or a portion of the Y chromosome is present [5]. The prevalence of Y-chromosome sequences in TS varied from 4.6% to 60%. The most frequently investigated genes were *SRY*, *DYZ3*, and *TSPY*, and the primary method of genetic assessment was polymerase chain reaction (PCR) [6]. The advantage of PCR is that it is inexpensive, sensitive, rapid, and it enables the tracking of various sequences of the Y chromosome simultaneously. The gonadoblastoma gene (*GBY*), which is located at Yp close to the centromere, is a gene that may cause gonadoblastoma [7]; however, the presence of the Y chromosome in TS patients increases the risk of developing gonadoblastoma in unilateral or bilateral gonads by 15-30% [8]. If any of the phenotypic female patients, including monosomy X TS or mosaic TS, possess the Y chromosome or Y-chromosome sequences, early prophylactic gonadectomy is strongly recommended to prevent germ cell tumor in the future (usually before 30 years old) [7]. TS patients are, therefore, good subjects for Y chromosome or Y-chromosomal sequence detection.

Many other techniques have been used to identify human chromosomal fragments or sequences, such as singlestrand conformational polymorphism (SSCP), fluorescence *in situ* hybridization (FISH), heteroduplex analysis, and electrophoresis. However, PCR is a simple, easy to perform, and cost-efficient technique that was determined to be the most favorable technique for use in this study. PCR can be performed as a single PCR to identify one single DNA fragment of various lengths. However, an efficient multiplex PCR that can simultaneously yield multiple DNA fragments of sequences of interest has been developed [9].

Simple, rapid, highly sensitive, and accurate PCR conditions for determination of 9 Y-chromosomal DNA fragments from DNA extracted from a peripheral whole blood specimen using 2 PCR conditions was developed in this study. Assay A (pentaplex PCR; 5 primer sets) and Assay B 4 primer sets) are each performed with specific cycle amplifications. Nine different Y-chromosome sequences representing 9 important loci on the Ychromosome were amplified, as follows: 4 loci on short arm (Yp), which were pseudoautosomal boundary region, Y-linked (*PABY*) located near Yp telomere [10-12], *SRY* located on Yp11.2 [13-14], *ZFY* located on Yp11.2 [10], and amelogenin Y (*AMGLXY*) located on Yp11 [15-18]; 1 locus on Y-centromere [11]; and, 4 loci on long arm (Yq), which were azoospermia factor (*AZFa*, *AZFb*, *AZFc*) located on Yq11.21 [18-20] and YD, and loci of heterochromatin located on Yq [21]. Amplifications of gene segments of a zinc finger and amelogenin revealing both Y- and X-specific bands were used for internal amplification control.

2. Materials and methods

2.1 Subject selection

This cross-sectional experiment study enrolled 20 consecutive TS patients who attended the outpatient clinic of the Gynecologic Endocrinology Unit, Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Patients with a sufficient collection of various peripheral blood chromosomal results were eligible for inclusion. Five normal female and 5 normal male DNA controls were obtained from our cytogenetic laboratory during the same study period. This study was approved by the Siriraj Ethical Committee on Research Involving Human Subjects (approval no. 69/2002). All patients and controls were aged ≥18 years, and all participants provided written informed consent to participate.

2.2 Cytogenetic analysis

Chromosome analyses were performed using peripheral blood lymphocytes by a standard cytogenetic technique. Metaphase chromosomes were also evaluated by Giemsa-Tripsin-Giemsa (GTG) banding, and at least 50 metaphases were analyzed by two cytogeneticists [22].

2.3 Molecular genetic analysis

2.3.1 DNA extraction

Genomic DNA from study subjects were extracted from 2-6 ml ethylene diamine tetra acetate (EDTA) peripheral blood leukocytes by proteinase K salting out methodology [23]. All DNA extractions were performed

by female technicians to avoid contamination by male DNA material. DNA concentration was assessed with NanoDrop 2000/2000c Spectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA)

2.3.2 Polymerase chain reaction (PCR) amplification

Nine different loci of Y-chromosome sequences located along the Y chromosome were selected for detection of Y-chromosome materials in this study. Primer sequences and the expected sizes of PCR products and references are summarized in Table 1 [10-21]. PCR products of 9 primer sets were assessed with two assays of PCR amplification. For Assay A, pentaplex PCR products consisted of loci *ZFX/ZFY*, *SRY*, *AZFa*, *AZFb*, and *AZFc*; and, the Assay B PCR products consisted of loci *PABY*, *AMGLXX/AMGLXY*, Y-centromere, and YD. A diagram showing 9 loci on the Y chromosome is shown in Figure 1.

Table 1 The Y-chromosome loci, primer sequences, product sizes, annealing temperatures, and references used in this study.

PCR assay	Y chromosome loci/ (Product name)	Primer name	Sequences 5'→3'	Product (bp)	Anneal T (˚C)	Ref.
Set A	Zinc finger/ (*ZFX/*ZFY)	ZFX/Y-F	5' ACC act GTA ctg ACT gtg ATT aca C 3'	495	55	[10]
		ZFX/Y-R	5' GCA ctt CTT tgg TAT ctg AGA aag t 3'	495	55	[10]
	*SRY/(*SRY*)*	SRY-F	5' GAA tat TCC cgc TCT ccg GA 3'	472	55	[13-14]
		SRY-R	5' GCT ggt GCT cca TTC ttg AG 3'	472	55	[13-14]
	*AZFa/(*AZFa*)*	sY84-F	5' AGA agg GTC tga AAG cag GT 3'	326	55	[18-20]
		sY84-R	5' GCC tac TAC ctg GAG gct TC 3	326	55	[18-20]
	*AZFb/(*AZFb*)*	sY134-F	5' GTC tgc CTC acc ATA aaa CG 3'	301	55	[18-20]
		sY134-R	5' ACC act GCC aaa ACT ttc AA3'	301	55	[18-20]
	*AZFc/(*AZFb*)*	sY255-F	5' GTT aca GGA ttc GGC gtg AT 3'	126	55	[18-20]
		sY255-R	5' CTC gtc ATG tgc AGC cac3'	126	55	[18-20]
Set B	Yp-terminal/(*PABY*)	PABY-F	5' CTG aga GTG gaa GTG tcg CAG 3'	1100	55	[10-12]
		PABY-R	5' GTA cta CCT tta GAA aac TAG tat TTT ccc 3'	1100	55	[10-12]
	Amelogenin/ (AMGLXX&*AMGLXY*)	AMGL-F	5' CTG atg GTT ggc CTC aag CCT gtg 3'	977(F) 977&788(M)	65	[15-17]
		AMGL-R	5' TAA aga GAT tca TTA act TGA ctg 3'	977(F) 977&788(M)	65	[15-17]
	Y-centromere/	Y1	5' ATG ata GAA acg GAA ata TG 3'	170	55	[11]
	(Y-Centromere)	Y2	5' AGT aga ATG caa AGG gct CC 3'	170	55	[11]
	Yq-terminal/(YD)	YD1	5' TCC act TTA ttc CAG gcc TGT cc 3'	154	55	[21]
		YD2	5' TTG aat GGA atg GGA acg AAT gg 3'	154	55	[21]

Note. *SRY;* sex-determining region Y, *AZFa-c;* azoospermia factor region a-c, Yp; short arm Y chromosome, *PABY;* pseudoautosomal boundary region Y, Yq; long arm Y chromosome, F; Female, M: Male, bp; base pair, T; temperature, Ref; reference

PCR Assay A: Pentaplex PCR using 5 primer sets of 5 loci, which were Yp/*ZFX/ZFY* (495 bp), Yp/*SRY* (472 bp), Yq/*AZFa* (326 bp), Yq/*AZFb* (301 bp), and Yq/*AZFc* (126 bp), was prepared. A total of 50 µl PCR reaction included 50 ng extracted DNA, 200 pmole of each forward and reverse primer, 25 µl of HotStarTaq Master Mix (containing 1x PCR buffer, 1.5 mM MgCl2, 200 uM dNTP), and 2.5 U HotstarTaq Polymerase. The thermal cycle was performed, as follows: initial activation of HotstarTaq Polymerase at 95 ˚C for 10 min, followed by 30 cycles of denaturation at 94 ˚C for 1 min, annealing at 54 ˚C for 40 sec and extension at 72 ˚C for 4 min, with final extension for complete termination at 72 ˚C for 5 min on a Perkin-Elmer GeneAmp PCR System 2400. The pentaplex PCR products were separated by 4% agarose gel electrophoresis and visualized by exposure to UV light after ethidium bromide staining.

PCR Assay B: Each tube of each loci product of Yp/*PABY* (1100 bp), Yp/*AMGLXX* (977 bp)/*AMGLXY* (788 bp), Y-Cen/Y (170 bp), and Yq/YD (154 bp) was prepared with a total 25 µl PCR reaction, 10 uM Tris (pH7.8), 1.5 mM $MgCl_2$, 200 uM dNTP, 200 pmole of each forward and reverse primer, 1.25 U Tag DNA polymerase, and 50 ng extracted DNA. Each PCR tube was simultaneously amplified on a Perkin-Elmer GeneAmp PCR System 2400. The thermal cycle was performed, as follows: initial denaturing 1 cycle at 95 ˚C for 5 min, followed by 35 cycles of denaturation at 94 ˚C for 1 min, annealing at 54 ˚C for 2 min and extension at 72 ˚C for 5 min, with final extension 1 cycle at 72 ˚C 5 min for complete termination. Each of the four PCR products was separated by 2% agarose gel electrophoresis and visualized by exposure to UV light after ethidium bromide staining.

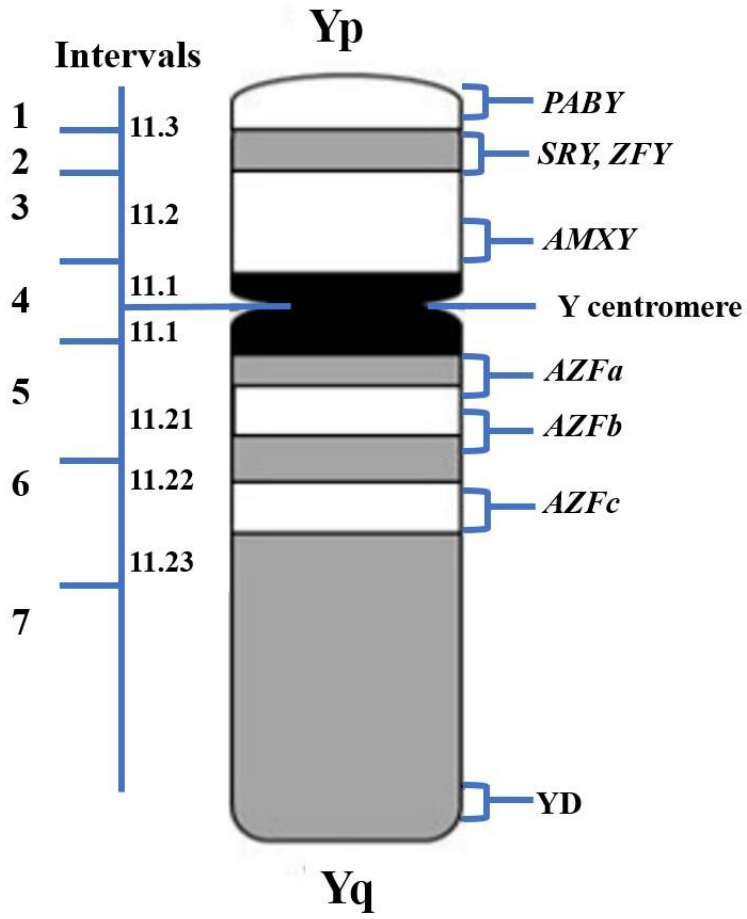

Figure 1 Schematic representation of human Y chromosome with 9 loci investigated in this study. *PABY*; the pseudoautosomal boundary region of Y, *SRY*; sex determine region on Y chromosome, *ZFY*; Zing finger on Y chromosome, *AMGLY*; amelogenin like sequence, Y-centromere, *AZFa-c*; azoospermia factor region a-c, YD; Yq-terminal. Adapted from the International System for Human Cytogenomic Nomenclature [24].

3. Results and discussion

Gel electrophoresis for rapid PCR product detection of human sequences of the Y chromosome from Assay A – the pentaplex PCR products of loci *ZFX/ZFY*, *SRY*, *AZFa*, *AZFb*, and *AZFc* are shown as Figure 2. The products from Assay B representing 4 or 5 PCR products of 5 loci – *PABY*, *AMGLXX/AMGLXY*, Y-Cen, and YD are shown in Figure 3. All TS patients and normal female controls showed positive PCR control products of *ZFX/ZFY* and *AMGLXX*. The conventional cytogenetic karyotypes and PCR products of 9 loci on the Y chromosome of 20 TS patients are shown in Table 2. Ten positive PCR products of 9 loci were present in patients #3 and #9 whose karyotypes were 45,X/46,XY and 45,X/46,X,+mar, respectively. The other 5 mosaic TS patients (patients #1, #8, #12, #13, and #20) showed positive results for only the two control PCR products mentioned earlier. For karyotype 46,X,+mar (patients #2 and #5), both patients showed the same results as for karyotype 46,X,i(X)(q10) (patient #10). Marker chromosomes (patients #2, #5, and #11) were confirmed by FISH with X-Y probe. The results of patient 11 showed positive for the Y chromosome, while those of patients #2 and #5 were negative. Interestingly,

patient #14 who has a karyotype of monosomy X (45,X) showed positive products of *PABY*, *SRY*, *ZFX/ZFY*, and *AMGLXX/AMGLXY*. Whereas, 8 other 45,X karyotype TS patients were positive for only the two control products of *ZFX/ZFY* and *AMGLXX*.

Prophylactic gonadectomy was performed in patients #3, #9, and #11, and pathological examination of their gonads showed testicular tissue. Patient #14 was 37 years old without sign of virilization, and with completely normal female external genitalia. This patient was scheduled to confirm for the short arm of the Y chromosome by Y probe with FISH technique, and planned to undergo gonadectomy if partial Y chromosome was positive, but she was lost to follow-up.

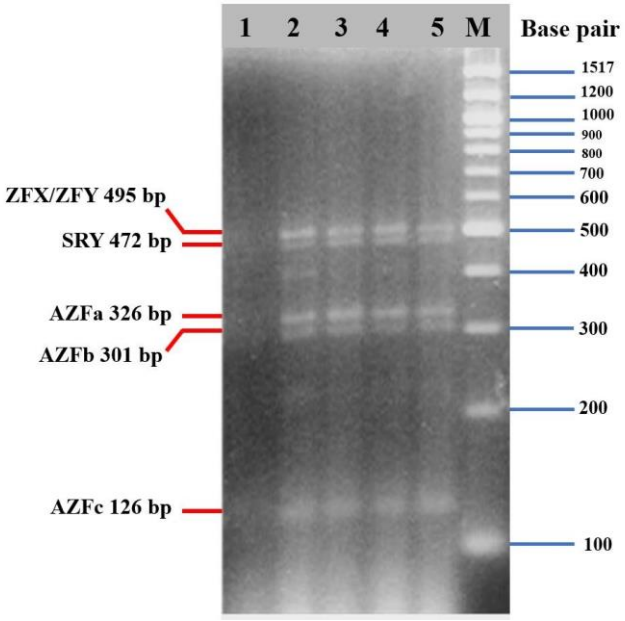

Figure 2 Pentaplex PCR products of Assay A, products of *ZFX/ZFY*, *SRY*, *AZFa*, *AZFb*, and *AZFc* separated by 4% agarose gel. Lane 1; water, lane 2; normal male (46,XY), lane 3; case #3 (45,X/46,XY), lane 4; case #9 (45,X/46,XY), lane 5; case #11 (45,X/46,X,+mar), and lane M; 100 bp DNA Ladders.

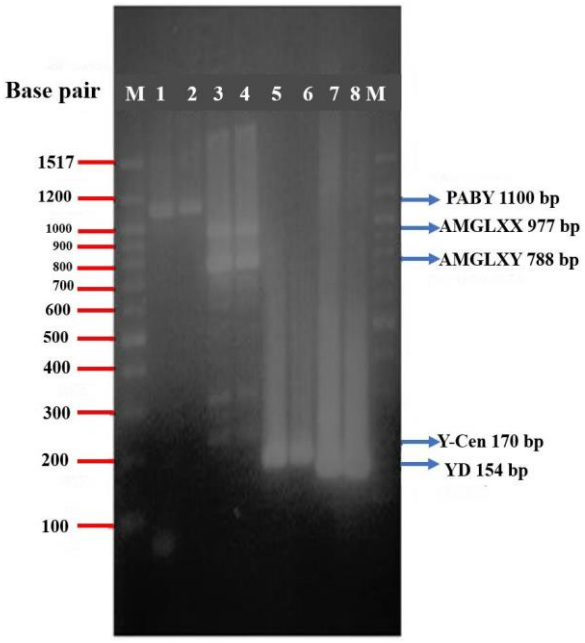

Figure 3 PCR products of Assay B, products of 4 regions; *PABY*, *AMGLXX/AMGLXY*, Y centromere and YD from male DNA control (46,XY) separated by 2% agarose gel. Lane M; 100 bp DNA Ladders, lane 1&2; *PABY*, lane 3&4; *AMGLXX/AMGLXY*, lane 5&6; Y centromere, lane 7&8; YD.

Table 2 Showed results of conventional cytogenetic karyotypes of consecutive 20 TS patients and their PCR products of 9 loci on the Y chromosome in this study.

Patient	Karyotype	*PABY*	*SRY*	*ZFX/ZFY*	*AMGLXX/ AMGLXY*	Y-Cen	*AZFa*	*AZFb*	*AZFc*	YD
TS1	45,X/46,i(X)(q10)	-	-	+	+/-	-	-	-	-	-
TS2	46,X,+mar	-	-	+	+/-	-	-	-	-	-
TS3	45,X/46,XY	+	+	+	+/+	+	+	+	+	+
TS4	45,X	-	-	+	+/-	-	-	-	-	-
TS5	46,X,+mar	-	-	+	+/-	-	-	-	-	-
TS6	45,X	-	-	+	+/-	-	-	-	-	-
TS7	45,X	-	-	+	+/-	-	-	-	-	-
TS8	45,X/46,XX	-	-	+	+/-	-	-	-	-	-
TS9	45,X/46,XY	+	+	+	+/+	+	+	+	+	+
TS10	45,X/46,i(X)(q10)	-	-	+	+/-	-	-	-	-	-
TS11	45,X/46,X,+mar	+	+	+	+/+	+	+	+	+	+
TS12	45,X/46,i(X)(q10)	-	-	+	+/-	-	-	-	-	-
TS13	45,X/46,X,der(X)t(X;?)(q28;?)	-	-	+	+/-	-	-	-	-	-
TS14	45,X	+	+	+	+/+	-	-	-	-	-
TS15	45,X	-	-	+	+/-	-	-	-	-	-
TS16	45,X	-	-	+	+/-	-	-	-	-	-
TS17	45,X	-	-	+	+/-	-	-	-	-	-
TS18	45,X	-	-	+	+/-	-	-	-	-	-
TS19	45,X	-	-	+	+/-	-	-	-	-	-
TS20	45,X/47,XXX	-	-	+	+/-	-	-	-	-	-

PCR is widely accepted as a technique for detecting Y-chromosome sequences from various sources of DNA, especially lymphocytes from peripheral blood. PCR is a simple and rapid process that yields a reliable and accurate result. Genomic DNA amplification, especially for clinical diagnosis, requires compliance with good clinical practice and strict quality control. Previously published data showed that Y-sequences spanning the whole Ychromosomal length, both Yp and Yq, could be divided into fragments. Many primer pairs, one pair for each single PCR product, have been reported [10-21]. Internal quality controls should be carefully established when implementing a diagnostic protocol according to Y-chromosomal sequence detection. Apart from only female technicians being involved in the laboratory process in this study according to the standard guideline, normal male and normal female samples have to be used as controls for DNA contamination during the entire procedure. Each set of PCR reactions from patient DNA samples should be performed in at least duplex or multiplex PCR including internal control loci. Multiplex PCR is also useful for distinguishing a false-negative or a false-positive result from a technical error via the presence of internal control sequences.

In this study, an appropriate internal sequence for PCR control in Assay A is the *ZFX/ZFY* gene producing a 495 bp product. For Assay B, PCR of amelogenin produces a single band of 977 bp in a female control, and two bands of 977 and 788 bp in a male control as the results of one same primer set amplification. However, to be more accurate and to avoid false results, a sample that contains all reaction components, including water instead of DNA, must be run with each set of primers as another internal control for detecting contamination. Evidence has been reported that fragments or sequences of the Y chromosome or a marker chromosome (45,X/46,X,+mar) were identified in TS. Those fragments or sequences sometimes could not be detected in routine standard karyotyping. However, most experts agree that routine testing with fluorescent *in-situ* hybridization (FISH), chromosomal microarray, or karyotype of buccal tissue/skin fibroblasts is not necessary, but may be considered in virilized girls in whom Y-chromosome presence was not demonstrated by a standard karyotype analysis [25]. Multiple sequences adjacent to the Y-centromere should be amplified using PCR techniques that are more sensitive than FISH to detect cryptic Y-material [26]. FISH was usually subsequently performed to confirm whether the fragment or the marker chromosome comprises Y-chromosome materials, but it is an expensive and time-consuming technique [27]. Although advanced PCR technique, such as real-time PCR, is now currently used for Y chromosome marker detection in TS patients [26], the conventional PCR is still used [28].

We found positive results of 4 loci on Yp in 1 of 9 monosomy TS patients (11.1%), and 9 loci spanning the whole Y chromosome in 3 of 11 mosaic TS patients (27.3%), which confirms the efficacy of the developed PCR conditions. Our results are comparable to those from previous reports that analyzed peripheral blood alone. The prevalence of Y sequences ranged from 4.6% to 18.5% [29]. However, in other studies where more than one tissue was evaluated, the prevalence may be higher [30]. According to our result, it can be concluded that molecular investigation is indicated for Y-chromosome sequences in TS patients, regardless of the karyotype, as a complement to the cytogenetic diagnosis. The advantage of our PCR conditions is that they are inexpensive, sensitive, rapid, and they enable the tracking of various sequences of Y-chromosome simultaneously. The limitation of our method is its inability to detect deletions or mutations, which is an investigation that requires a

different laboratory technique. Our method may also be applied for prenatal and neonatal sex determination, determining regions related to azoospermia or oligospermia causing male infertility.

4. Conclusion

PCR yielding 9 loci spanning through short arm (Yp), Y centromere, and long arm (Yq) of chromosome was developed. These PCR conditions serve as a rapid and effective method for identifying Y-specific DNA sequences including SRY in TS.

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