Detection of Y chromosome material in patients with Turner syndrome by molecular analysis

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Detection of Y chromosome material in patients with Turner syndrome by molecular analysis

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<ABSTRACT>

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Turner syndrome (TS) is a disorder characterized by short stature, gonadal failure and other endocrine or congenital abnormalities. Detecting the presence of Y chromosome material in TS without overt Y-mosaicism is very important because the patients with Y chromosome fragments have an increased risk for the development of gonadoblastoma. The aim of this study was to investigate the presence of Y chromosome material using Y-specific sequences in 130 Korean patients with TS, who had not shown any Y chromosome fragments analyzed by the cytogenetic method. Fourteen Y-specific sequences were amplified by polymerase chain reaction (PCR), using genomic DNA obtained from peripheral blood lymphocytes, to detect cryptic Y chromosome material. The PCR analysis demonstrated that 8 patients among 97 patients without overt Y chromosome (8.3 %) have hidden Y chromosome material, suggesting that PCR may be a more sensitive method than classical cytogenetic analysis to detect hidden Y chromosome material in patients with TS. Moreover, there is no difference in clinical

characteristics, including virilization between patients with and without Y chromosome material. These results suggest that the detection of hidden Y chromosome material by sensitive methods, such as PCR, should be included in the initial evaluation of all patients with TS to calculate the future risk of gonadoblastoma and its proper management.

Keywords: Turner syndrome, PCR, Y chromosome-specific sequences, gonadoblastoma

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I. INTRODUCTION

Turner syndrome (TS) is a chromosomal disorder characterized by short stature, ovarian failure and other endocrine abnormalities or congenital anomalies[1,2]. The incidence of TS is one in 1500-2500 female births[2,3]. It has been known that one in 50-100 children with short stature are diagnosed with TS[4]. About half of all patients with TS have a 45,X karyotype and the others have only a partial X chromosome or mosaicism[2,5]. In general, it has been reported that approximately 5% of patients with TS have Y chromosme material in cytogenetic analysis[2,6]. Many studies, however, have reported that 4-61% of patients with TS have incompletely derived Y chromosome material[1,7].

The presence of Y chromosome material is very important because Y chromosome-positive patients have an increased risk for the development of gonadoblastoma or other ovarian tumors. Some authors reported that the risk of development of gonadoblastoma in patients with TS is

more than 30%. Therefore, prophylactic gonadectomy has conventionally been recommended in Turner patients with Y chromosome[1,2,6,8,9]. To calculate the risk for the development of gonadoblastoma, an accurate method to detect Y chromosome material is essential. Many investigators have noted the low detection rate of Y chromosome material by traditional cytogenetic examination and have suggested the use of more sensitive methods, such as polymerase chain reaction (PCR) to detect hidden mosaicism[10-13].

In this study, the presence of hidden Y chromosome material was investigated using fourteen Y-specific sequences amplified by PCR in 97 Turner patients without overt Y chromosome by the cytogenetic method.

II. MATERIALS AND METHODS

1. Subjects

A total of 97 patients with TS were included in this study. These patients were diagnosed with TS by conventional cytogenetic technique and followed-up at the division of pediatric endocrinology in Severance Children's Hospital. The patients proved to have Y chromosome in cytogenetic analysis were excluded in the study. The patients ranged in age from 4 to 36 years. Medical records of clinical characteristics including short stature, delayed puberty, virilization, and other abnormalities as well as conventional chromosome results were reviewed. Blood sampling was done after written informed consent was obtained from the parents and patients. This study was approved by the Institute Review Board of Severance Hospital.

2. Methods

Cytogenetic analysis were performed in peripheral blood lymphocytes. At least 30 metaphases were analyzed by Giemsa-Tripsin-Giemsa banding for each patient.

Genomic DNA was extracted from whole blood samples obtained from 97 patients by QIAamp

DNA Blood Midi Kit (Qiagen, CA, USA) according to the Manufacturer's instructions. To confirm PCR specificity, blood samples from four healthy men and women were used for positive and negative control groups. Fourteen Y chromosome specific sequences located in both arms of the Y chromosome were used to detect hidden Y chromosome material by PCR analysis. Primer sequences were designed as described previously with minor modifications[2]. The Primer3 on-line software (www.ncbi.nlm.nih.gov and www.broad. mit.edu/cgibin/primer/primer3 www.cgi) which is widely used program for primer design was used for the analysis in this study. Firstly, DNA template sequences and product size range were input and Primer3 was utilized to design candidate primers for each of the DNA template sequences. And then evaluation of primer specificity with dimer examnination was performed by this program. Finally optimal primer set combinations were chosen for the study. Table 1 summarized primer sequences and their charateristics. PCR was performed with 20 ul of PCR amplification reaction mixture containing 10x Ex Taq DNA polymerase buffer with 2.5 mM of each dNTP, 0.5 M of each primer, 500 ng of DNA, and 2.5 units of Taq DNA polymerase (Takara Bio, Otsu, Japan). Amplification was performed in duplicate with the ABI 7300 system (Applied Biosystems, CA, USA) with the following profile: 94℃ for 5 min, 35 cycles of 95℃ for 30 seconds, 50℃ for 30 seconds (annealing temperature for each primer was summarized in Table 1), 72℃ for 30 seconds and final exyension at 72℃ for 10 min. PCR products were separayed by 1% agarose gel electrophoresis. All DNA extractions and PCR reactions were performed by a female researcher to avoid the risk of male DNA contamination and repeated more than 3 times.

Table 1. Primer sequence, annealing temperature, and product size

Primer	Sequence	Annealing temperature (℃)	Product size (bp)
PABY	5'-TCTCGATCTCCTGACCTCGT-3'	60	244
	5'-ATTGGGGCTTTGAAGGAACT-3'		
SRY	5'-TACAGGCCATGCACAGAGAG-3'	55	179
	5'-TCTTGAGTGTGTGGCTTTCG-3'		
DYZ1	5'-ATTGGAGTCCGTACCAGTCG-3'	53	181
	5'-TGAAATGGAATCGAACCACA-3'		
DYS231	5'-GGGATTGCAGAGAGCAAAAG-3'	55	159
	5'-GCCGTGTGCTGGAGACTAAT-3'		
DYS209	5'-TTGGTTCCATGCTCCATACA-3'	55	180
	5'-CTCCGAATGTTGCTCCAAAT-3'		
YRRM	5'-GAGGGCCTCGGATGTCTTAT-3'	55	224
	5'-TACCACATGCTTCACGAGGA-3'		
DYS224	5'-GTCTGCCTCACCATAAAACG-3'	55	152
	5'-ACCACTGCCAAAACTTTCAA-3'		
AMGY	5'-GGGCCCAGGACTCTATTTTC-3'	62	274
	5'-GCAGTGAGCTGAGATTGTGC-3'		
DYS273	5'-CTCTACCTCCTTCCCCCAGT-3'	55	191
	5'-GGAGGCTTCATCAGCAAGAC-3'		
DYS280	5'-CCCCATAATGACATCAGCCTA-3'	55	235
	5'-GTTGAGCCGGTCAAGAAAAA-3'		
DYS1	5'-CACTGCCCTAATCCTAGCACA-3'	55	127
	5'-TGGTCATGACAAAAGACGAA-3'		
DYS218	5'-AGGCTAGGCTCACAAACGAA-3'	55	201
	5'-CCAGCCCATTTAATCAAGGA-3'		
DYS14	5'-GGCTTCTCATTCCACTCCAA-3'	46	206
	5'-CCTCTTCAGGTGGCTTCATC-3'		

III. RESULTS

The cytogenetic analysis showed 45, X in 36 patients (37%) and other karyotype composition in 61 patients (63%) (Table 2). Mosaicism comprised 41% of the total subjects. PCR analysis using different fourteen Y chromosome specific sequences detected hidden T chromosome material in 8 patients (8.3%) among 97 Turner patients without overt Y chromosome by the cytogenetic method. Table 3 summarized the results of PCR analysis.

Of these 8 patients, 2 patients (#1,4) were positive for all 14 sequences. Patient #52 was positive for 12 sequences and negative for DYZ1/DYS1. Patient #101 was positive for 9 sequences and negative for DYZ1, DYS209, DYS224, DYS1, and DYS218. Patient #71 was positive for just two sequences, DYZ1 and DYS218. 3 patients (patient #122,123,133) were positive for only one sequence, DYZ1 (Figure 1).

Table 2. Distribution of the karyotype in 97 patients with Turner syndrome

Karyotype	n	%
45X	36	37
46X,i	16	17
45X/46XX	13	14
45X/46X,+mar	9	9
45X/46X,i	7	7
46X del	5	5
47XXX/45X	5	5
45X/46X,r	4	4
47XXX/45X/46XX	2	2
Total	97	100

Table 3. PCR analysis results of 8 patients positive for Y chromosome material[1]

Patient	Karyotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14
#1	46XX/45X	+	+	+	+	+	+	+	+	+	+	+	+	+	+
#3	46,X,del(x)(q11)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
#40	45X/46,X,+mar	+	+	-	+	+	+	+	+	+	+	-	+	+	+
#52	45,X	-	-	+	-	-	-	-	-	-	-	-	+	-	-
#70	45, X	+	+	-	+	-	+	-	+	+	+	-	-	+	+
#82	45X/46,X,i(Xq)	-	-	+	-	-	-	-	-	-	-	-	-	-	-
#83	46,X,i(Xq)	-	-	+	-	-	-	-	-	-	-	-	-	-	-
#89	45X/46,X,i(Xq)	-	-	+	-	-	-	-	-	-	-	-	-	-	-

1 Y chromosome-specific sequences are numbered as follows: 1, PABY;, 2, SRY;, 3, DYZ1;, 4, DYS231;, 5, DYS209;, 6, YRRM;, 7, DYS224;, 8, AMGY;, 9, DYS273;, 10, DYS280;, 11, DYS1;, 12, DYS218;, 13, DYS14;, and 14, DYZ3. A positive result is indicated by + and a negative result is indicated by -.

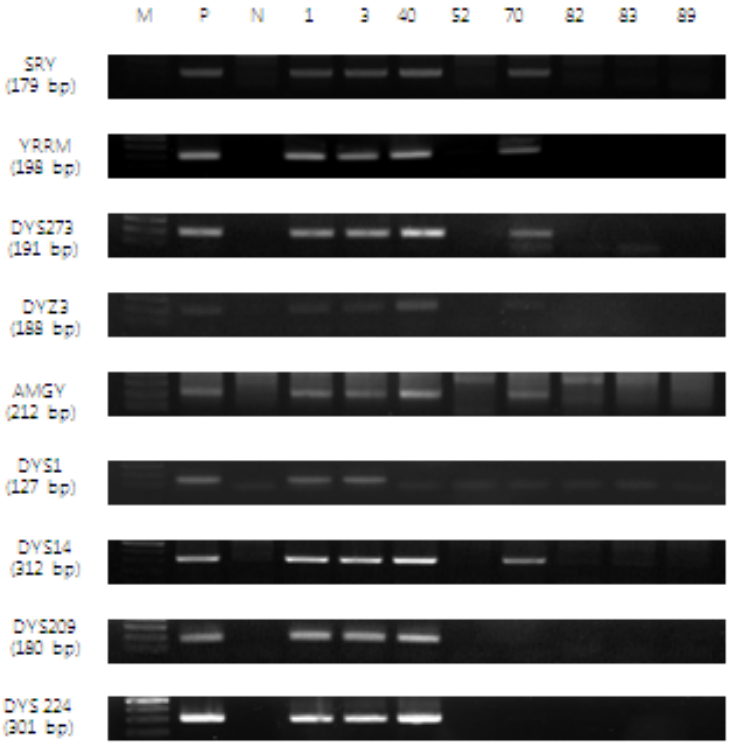

Figure 1.Detection of Y chromosome material in the positive group using polymerase chain reaction. M,100bp marker;, P, positive;, N, negative. Arabic numerals represent the number of patients.

Ninety-seven patients were classified into two groups according to the PCR results. Patients positive for at least one Y chromosome-specific sequence were considered positive (8 patients); those who were negative for all sequences represented the control group (89 patients). The median ages of the positive and control groups were 23.4 and 17.0 years, respectively. The height standard deviation scores (SDS) in the positive and control groups were -2.26±0.9 (mean ± SD) and -1.84±1.01, respectively, and weight SDS were -0.20± 1.03, and -0.67±1.42, respectively. BMI in the positive and control group were 22.83 ± 3.18 and 20.33 ± 4.19, respectively.

Additionally, Various clinical characteristics between the two groups were compared as follows: short stature below the third percentile for age and sex, a dysmorphic feature such as a webbed neck, cubitus valgus, osteopenia or osteoporosis diagnosed by bone densidometer, delayed puberty, cardiac abnormality based on echocardiogram or cardiac MRI, diabetes mellitus or impaired glucose tolerance, thyroid disease, virilization such as clitomegaly, and the development of ovarian tumor such as gonadoblastoma or dysgerminoma.

The characteristics of 8 patients in positive group are follows; patient #1 had received clitoroplasty in the neonatal period for ambiguous genitalia, and prophylactic ovareictomy at the age of 11, followed by revealing dysgerminoma). Besides, she had short stature, osteopenia and delayed puberty. Patient #4 had short statue, delayed puberty, and impaired glucose tolerance but showed no sign of virilization. Patient #52 had a osteopenia, delayed puberty and impaired glucose tolerance. Patient #71 had a ostepenia. Patient #101 had a short stature. Patient #122 has been treated for short stature and delayed puberty. Patient #123 had short stature, osteopenia, delayed puberty and impaired glucose tolerance. Patient #133 had short stature, osteopenia and delayed puberty. Among the control group without Y chromosome material, one patient had ovarian dermoid cyst with hemorrhagic infaction, followed by laparoscopic salpingo-oophorectomy. She had a karyotype of 47XXX/45X and virilization was not observed. This ovarian cyst was not considered as a development of true ovarian tumor. There was no statistically significant difference between the two groups with regard to these clinical findings (Table 4).

Table 4. Clinical comparison[1] of patients positive for Y chromosome material positive and the control group*

	Control group	Y material positive group
Number	89 (%)	8 (%)
Age (years)	17.0±8.5	23.4±9.4
Height SDS	-1.84±1.01	-2.26±0.9
Weight SDS	-0.67±1.42	-0.20± 1.03
BMI (kg/m^2)	20.33±4.19	22.83±3.18
Short stature	73 (82.0)	6 (75.0)
Dysmorphic figure	7 (12.7)	1 (8.3)
Osteopenia	11 (12.4)	0 (0)
Delayed puberty	51 (57.3)	6 (75.0)
Cardiac anomaly	17 (19.1)	0 (0)
DM or IGT	9 (10.1)	2 (25.0)
Thyroid disease	13 (14.6)	1 (12.5)
Virilization	0 (0)	1 (12.5)
Ovarian tumor	0 (0)	1 (12.5)

*No statistical significance was found between the two groups in all items ($p>0.05$)

1 Age, height SDS, weight SDS, and BMI are presented as mean ± standard deviation. All other items including short stature are presented as the percentage of total patients (%).

SDS, standard deviation score, ; BMI, body mass index

IV. DISCUSSION

The diagnosis of TS can be made by the presence of characteristic physical features in phenotypic females associated with complete or partial absence of the second sex chromosome, with or without cell line mosaicism[13]. Therefore, all individuals with clinical features suspected TS should have a karyotype performed. A standard 30-cell karyotype is recommended by the American College of Medical Genetics and identifies at least 10% mosaicism with 95% confidence[14]. In case of strong suspicion of TS clinically, although a subject has a normal blood karyotype, a second tissue, such as skin or oral epithelial cell, may be examined. In 2007, Turner Syndrome Study Group recommended that testing for Y chromosome material should be performed in any TS patients with a marker chromosome, which is a sex chromosomal fragment of unknown origin, and with the presence of virilization [15]. In addition, this guideline recommended that routine testing for SRY or the presence of Y chromosome material in 45, X individuals without masculinization is not clinically warranted at present. It also has been stated that additional investigation about the prevalence and clinical significance of cryptic Y material detected only by fluorescence *in situ* hybridization (FISH) or DNA analysis in patients without virilization or a marker chromosome should be exploited. Recently, many studies demonstrated that Y chromosome material in patients without overt Y chromosome by the cytogenetic method can be identified by FISH or molecular analysis, even though they are not virilized or do not have a marker chromosome, suggesting that it is necessary to screen the hidden Y chromosome material in all patients in TS.

In this study, molecular analysis was performed in patients with TS by PCR using 14 Y chromosome-specific sequences. PCR analysis detected hidden Y chromosome material in 8 patients (8.3%) among 97 Turner patients without overt Y chromosome by the cytogenetic method. The results of previous studies are generally consistent with our findings. In a Turkish study, 5% of patients with a pure 45,X karyotype had Y-specific material by PCR[2]. One

Mexican study identified Y-specific material in 9.3% of patients with a 45,X karyotype[1], and a Russian study reported more than 30% Y chromosome-material detection rate in patients with this karyotype[10]. Overally, previous studies reported that the frequency of Y chromosome material in patients with TS varies from 0% to 60% [2, 5, 16-19]. The variable results about the frequency of Y sequences may come from the differences of the molecular methodology, the ethinity, tissues tested, selection of Y chromosome-specific primers, and the number of the patients with mosaic marker chromosome[2].

In this study, the detection rate of Y-specific material was 8.3% in patients without overt Y mosaicism by conventional karyotyping, supporting previous reports describing the limitations of conventional cytogenetic analysis. Conventional cytogenetic analysis uses the metaphase chromosome from peripheral blood lymphocytes. This method misses Y chromosome derivatives when there is a low degree of Y chromosome mosaicism[20, 21]. For this reason, many researchers have emphasized the need for a more sensitive method to detect Y chromosome material. Turner syndrome patients with Y chromosome material are clinically undistinguishable from those without a Y chromosome. Indeed, there was no statistically significant differences between the group positive for Y chromosome material and control group with respect to clinical factors[10-12, 22-24]. Moreover, only one case shows virilization and one case has marker chromosome among the group positive for Y chromosome in our study, suggesting that all patients with TS should be screened to detect the hidden Y chromosome material.

Turner patients with Y chromosome material are at risk for the development of germ cell tumors such as gonadoblastoma or dysgerminoma, therefore more accurate method to detect Y chromosome material is needed. Gonadoblastoma is a neoplasm composed of a mixture of gonadal elements, such as large primordial germ cells, immature Sertoli cells or granulosa cells of the sex cord, and gonadal stromal cells. The pathogenesis of gonadoblastoma is unclear[1,2,6]. One hypothesis suggests that the gonadoblastoma locus on the Y chromosome (GBY) may have

oncogenetic alterations in dysgenetic gonads, including those of Turner syndrome. The incidence rate of gonadoblastoma in Turner patient with Y chromosome material is as high as 30% and may be higher in patients receiving hormonal replacement for ovarian failure.

In this study, 1 of 8 patients with Y chromosome material exhibited virilization. In conventional cytogenetic analysis, this patients (patient #1) had a 46,XX/45,X karyotype; PCR, however, gave a positive result for all Y-specific sequences tested. She had received clitoroplasty in the neonatal period for ambiguous genitalia, and after developing dysgerminoma at the age of 11, finally received ovariectomy.

Interestingly, patient #71 had a karyotype of 45,X and was negative for SRY but was positive for DYZ1 and DYS218. This result suggests the significance of using diverse sequences, other than SRY, to detect Y chromosome material.

PCR analysis using various Y-specific sequences seems to be a reliable method to confirm the presence of Y chromosome-derived material in patients with Turner syndrome and may be help prevent gonadoblastoma in Turner patients.

V. CONCLUSION

The presence of Y chromosome material in Turner patients increases the risk of gonadoblastoma. For this reason, it is important to detect hidden Y chromosome material using a sensitive and reliable method. Various data including our results suggest that molecular analysis, such as PCR, is helpful to detect hidden Y chromosome material and recommended in all patients with Turner syndrome.

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ABSTRACT(IN KOREAN)

논문제목

터너 증후군 환자에서 분자학적 분석을 이용한 Y 염색체 검출

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현세은

터너증후군은 저신장, 난소부전과 함께 기타 내분비적이상과 심혈관계 기형을 동반하는 염색체 질환이다. Y 염색체를 가진 터너증후군 환자는 생식모세포종의 발생 위험이 증가하기 때문에 Y 염색체 물질 존재 여부를 확인하는 것은 매우 중요하다. 이 연구에서는 기존의 고전적 세포유전학 방법에 비하여 모자이시즘을 확인하는데 더 민감도가 높은 PCR 기법을 사용하였다. 고전적 세포유전학 방법으로 Y 염색체가 발견되지 않았던 97 명의 터너증후군 환자를 대상으로 14 개의 Y 염색체 특이 염기서열을 이용하여 PCR을 시행한 결과 8 명(8.3%)의 터너증후군 환자에서 Y염색체 물질을 확인하였다. Y염색체 물질이 존재하는 군과 존재하지 않는 군 사이에 임상 양상의 차이는 발견되지 않았으나 Y염색체 물질이 확인된 8 명의 환자 중 남성화를 보인 경우가 1 명 나타났으며, marker chromosome을 가진 경우가 1 명에서 관찰되었다.

이상의 결과에 근거하여 남성화나 marker chromosome이 있는 경우 뿐만 아니라 모든 터너증후군 환자에서 진단 시 숨겨진 Y 염색체를 확인하는 것이 필요하다고

생각되며, 터너증후군 환자에서 숨겨진 Y 염색체를 검출하는데 있어 기존 세포유전학 방법보다 민감도가 높은 PCR 사용이 보다 더 유용하다고 생각된다.

핵심되는말: 터너증후군, PCR, Y 염색체 특이 염기서열, 생식모세포종