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# Molecular Detection of 45, XO Turner's Syndrome in Short Stature Girls

**Neelam Saba and Saba Irshad***
*Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore.*

**Abstract.-** Turner's syndrome is a disorder in human females characterized by the presence of one normal X chromosome and the complete or partial loss of the second X chromosome. Turner's syndrome is usually diagnosed by karyotyping which is time-consuming, expensive and unfeasible for population screening. The present method of molecular detection was based on the ability of *Hpa*II, a methylation sensitive endonuclease, to induce the cleavage of non-methylated DNA in the active X-allele. Genomic DNA was obtained from 30 affected females with clinical symptoms of Turner's syndrome. After digestion with *Hpa*II, DNA was amplified with primers specific for a part of exon 1 of the androgen receptor (AR) gene and for GAPDH control gene. Normal females with a second methylated X chromosome escaped from *Hpa*II digestion and produced a band corresponding to AR gene amplification. 45, XO patients have just one active non-methylated X chromosome, completely digested by *Hpa*II, thus there was no amplification of the AR gene. Using this three-step diagnostic procedure, 45, XO karyotype was detected in 6 cases among 30 patients. Later on these samples were confirmed by karyotyping. One case was found with isochromosome pattern of chromosomal constitution, while in two other cases 45, XO/46, XX mosaicism was detected after karyotyping. So 45, XO monosomy was detected in 6 among 9 Turner patients (6/9). It was concluded from this study that the method of molecular detection is cost effective, rapid and more appropriate to detect Turner's syndrome as compared to karyotyping.

**Key words**: Turner's syndrome, X-chromosome methylation, mosaicism, methylation sensitive, isochromosome, active X-allele.

## INTRODUCTION

Turner's syndrome (TS), is a chromosomal disorder in human females characterized by the absence of all or part of a normal second sex chromosome, leads to a constellation of physical findings that often includes congenital lymphedema (edema of hands and feet), short stature, and gonadal dysgenesis (Turner, 1938; Ford *et al*., 1959). Turner's syndrome occurs in 1/2500 to 1/3000 live-born girls (Nielsen and Wohlert, 1991).

In 50% of TS cases, only one of the X chromosomes is inherited as a result of non-disjunction during parental gametogenesis, leaving a monosomy X genotype (45, XO) in the fetus. Of the females with TS that are not monosomic, approximately 5% to 10% have an isochromosome X, which results from duplication of the long arm of one X chromosome with loss of the short arm of that X chromosome (46, Xi (Xq)). Most of the rest have mosaicism for 45, XO, with one or more additional cell lineages *i.e.* 45, XO/46, XX or 45, XO/46, Xi (Xq) (Virginia *et al*., 2004).

Short stature and ovarian failure are the most common clinical presentations in a phenotypic female. The phenotypes of Turner's syndrome result from haplo-insufficiency (single copy of the X chromosome) of genes expressed in both sex chromosomes (Zinn and Ross, 2001). The more commonly reported features of Turner's syndrome include short stature, lymphedema, webbing of the neck, low posterior hairline, low set ears, nail dysplasia, broad chest, widely spaced nipples, anomalous often prominent ears and micrognathia (abnormal smallness of the jaws). Less commonly reported features include ptosis (drooping of eyelids), hypertelorism (abnormal distance between two organs), vertebral and other skeletal anomalies and single transverse palmar crease (Savendahl and Davenport, 2000; Ranke and Saenger, 2001).

Genes related to TS are located in the short arm of the X-chromosome, and are able to escape methylation, determining the expression of both alleles (Kosho *et al*., 1999). Despite Barr body formation of the second X chromosome in normal

* Corresponding Author: saba@ibb.pu.edu.pk, saba_dr@hotmail.com
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females, 15% to 20% of X chromosome genes on the "inactive" X are, in fact, transcribed (Brown and Greally, 2003). An example of this phenomenon is the short stature homeobox-containing (SHOX) gene, which is currently the only specific gene known to contribute to the TS phenotype (Blaschke and Rappold, 2000). All individuals apparently need biallelic SHOX expression to attain their full height potential. The absence of 1 allele results in a substantial loss in adult height. Similarly, ovarian failure may be due to inadequate dosage of X linked genes (Krauss *et al*., 1987) or incomplete meiotic-chromosome pairing (Burgoyne and Baker, 1985).

45, XO cells develop abnormal parity of sexual chromosomes during meiosis and functioning haploinsufficiency of genes located in the short arm. Several genes related to the clinical signs observed in TS patients have been mapped to this region, including short stature-related genes (Xp11.2-p22.1), ovary function regulatory genes (Xq13-q16) and genes related to lymphatic anomalies (Xp and Yp pseudoautosomal region) (Zinn and Ross, 2001). To date, there are no genetic data available which would explain the soft tissue and visceral stigmata in Turner's syndrome, such as lymphedema, webbing of the neck, and congenital heart failure. It has been proposed that, in primary lymphatic hypoplasia (an incomplete or arrested development of lymphatic vessels), tissues and organs in the vicinity of the affected lymphatic system are secondarily affected (Vittay *et al*., 1980; Ogata *et al*., 2000).

Females with the 45, XO karyotype are usually diagnosed earlier than those with mosaicism because their physical features tend to be more pronounced (Sybert and McCauley, 2004). Lymphedema, manifesting as puffy hands and feet, short stature, and delayed or absent puberty are often key indicators in many of these cases (Savendahl and Davenport, 2000). Other less specific abnormalities in infants with TS include frequent spitting, vomiting, difficulty in latching and sucking, gastrointestinal reflux, and failure to thrive, mostly caused by anatomical abnormalities of the oropharynx (oral part of the pharynx**)** (Sybert, 2005).

Karyotyping is used routinely to diagnose TS. It includes cell culture, which is one of the disadvantages of this technique, increasing the final cost, delaying the results and requiring blood samples in large amount. These limitations often prevent the use of karyotyping as a routine test to investigate patients with short stature. Main purpose of the present study is to develop a cost effective diagnostic tool that can be used to identify TS patients with 45, XO karyotype.

## MATERIALS AND METHODS

*Patients and methods*

The present study was carried out on girls (n=30: all girls with short stature; age range 1-20 years) with suspected Turner's syndrome presenting at the Pediatric Genetics Department of the Children Hospital and Institute of Child Health (CH&ICH) Lahore.

After informed consent of hospital authorities sampling was done in 7 months period (June 2007-December 2007) on every Monday. Clinical examination included detailed history, presenting complaints, family history and socio-economic status. Complete information about the patients was recorded on the proforma. Sampling involved many visits to the hospital during which 5 ml blood was collected in sterile sodium -EDTA vacutainer tubes. Blood samples from normal individuals were also collected from the students of Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore.

Genomic DNA was obtained from peripheral blood samples. Extraction was done through salting out procedure (Miller *et al*., 1988).

After extraction approximately 1µg of DNA was digested with *Hpa*II restriction endonuclease (Fermentas), for 16 h at 37°C in order to induce DNA fragmentation in non-methylated regions. Control experiments for the digestion were performed using DNA and buffer without *Hpa*II endonuclease. Each reaction was carried out in 16µl of nucleases free water, 0.5µl of *Hpa*II enzyme, 2µl of buffer and 2µl of diluted DNA solution.

After digestion a PCR reaction was carried out using a pair of primers specific for the amplification of a part of exon 1 of androgen receptor (AR) gene (accession number GDB: 120556; OMIM entry: 313700) located on the long

**Table I.- The sequence of oligonucleotide primers.**

| Direction of primer | Sequence of primer | Length (bp) | nM | Tm$^{\alpha}$ | Product size (bp) |
|---|---|---|---|---|---|
| Primer F (AR*) | 5′CTGAGCAAGAGAAGGGGAGGCGGGGTAAGGGAA GTAGGTGGA 3′ | 42 | 35.3 | 75.0 | 419bp |
| Primer R (AR) | 5′CGACTGCGGCTGTGAAGGTTGCTGTTCCTCATC CAG 3′ | 36 | 52.1 | 72.2 | |
| Primer F (GAPDH**) | 5′TCTCCAGAACATCATCCCTGCCAC 3′ | 24 | 42.2 | 62.1 | 419bp |
| Primer R (GAPDH) | 5′TGGGCCATGAGGTCCACCACCCTG 3′ | 24 | 49.3 | 67.2 | |

*, Androgen receptor gene; **, Glyceraldehyde phosphate dehydrogenase; α: Melting temperature

arm of the X chromosome (region Xq11-q12) (Fig. 1). PCR was also carried out on normal male and female blood samples after *Hpa*II digestion. Control reaction for each sample was set using GAPDH-pseudogene primers in a separate tube. The forward and reverse primers (Gene-Link) are given in Table I. To perform PCR reaction, a master mix was prepared containing water, buffer (10X PCR buffer with $(NH_4)_2SO_4$), dNTPS (Fermentas), reverse and forward primers (0.4µM each) and Taq DNA polymerase (2.5U) (Fermentas) in a single tube, which can then be aliquoted into individual tubes containing template DNA. Optimized concentration of $MgCl_2$ (Fermentas) was then added. The mixtures were heated to 95°C for 5 minutes for initial denaturation and then subjected to 35 cycles of amplification in a thermocycler (Eppendorf). The cycles consist of denaturation at 95°C for 1 minute, 3 min annealing at 58°C and 3 min extension at 72°C, followed by 10 min at 72°C for final extension.


Fig. 1. Region on X-chromosome indicating the position of HUMARA gene.

A buffer (3µl of 6X loading dye) was then added to the PCR product and submitted to electrophoresis in a 1% agarose gel, containing ethidium bromide. Agarose gel electrophoresis was carried out in 1X TBE buffer (Sambrook and Russel, 2001). Intensity of the amplified bands was checked with ethidium bromide under the UV light in Gel Documentation System (Gene Snap gel system).

## RESULTS

A total of 30 DNA samples extracted from peripheral blood were amplified after *Hpa*II digestion. Amplification of the band corresponding to the GAPDH-pseudogene was observed in all 30 cases, confirming DNA quality after digestion (Fig. 2). Analysis of 30 samples gave normal results in 24, corresponding to AR gene amplification which excludes 45, XO TS in these samples. AR gene amplification indicated the presence of a second, methylated X chromosome that was not digested by *Hpa*II enzyme.

Remaining 6 samples (20%), did not show a band corresponding to AR gene amplification after *Hpa*II digestion though they gave GAPDH-pseudogene amplification (Fig. 3), indicating that only one active non-methylated X chromosome (45, XO) was present in these samples.

Normal male and female DNA was also amplified after *Hpa*II digestion. Band corresponding to AR gene amplification was only observed in female case while in case of male there was no band

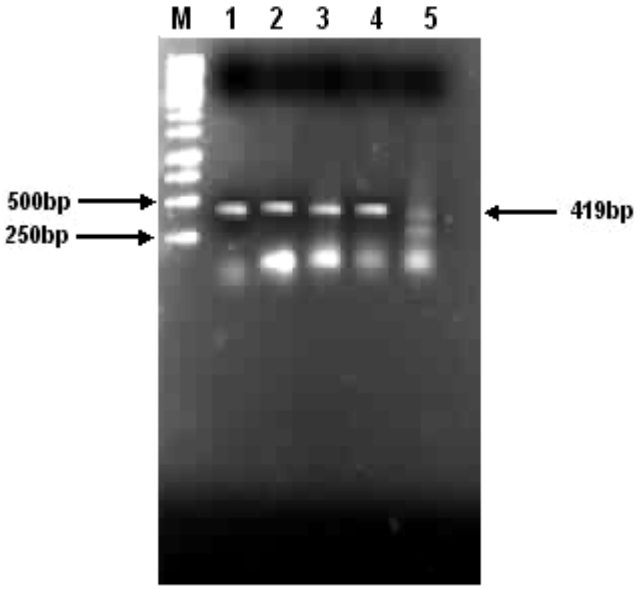


Fig. 2. PCR amplification of a segment of GADPH- pseudogene used as a control. Lane 1-4, 419 bp band correspond to GAPDH-psudogene amplification. Lane 5, non specific amplification with GAPDH primer. Lane M= molecular weight marker.

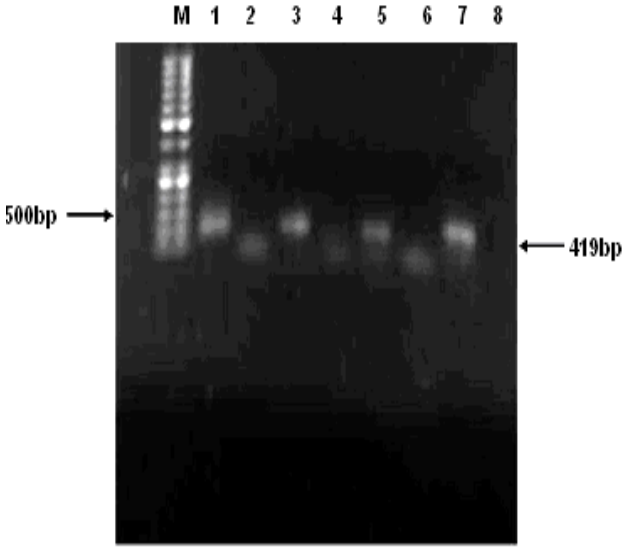


Fig. 3. PCR products of four patients screened for TS after DNA digestion with *Hpa*II. Lanes 1-8, a pattern of 45, XO amplification is shown. 1st lane correspond to segment of GAPDH amplification which was used as a control and 2nd lane correspond to pattern of AR amplification in 45, XO Turner females from 1-8. Lane M=molecular weight marker.

when PCR was carried out on *Hpa*II digested samples (Figs. 4, 5, respectively). Later on these samples were confirmed by karyotyping. After karyotyping mosaic Turner karyotype was observed in 3 cases (one case with 46, XX/46, Xi (Xq) and two with 46, XX/45, XO chromosomal constitution) in remaining 24 samples. These cases were presented due to absence of menstruation (primary amenorrhea). Dysmorphic features were not very much prominent. Uterus was of infantile type and ovaries were found abnormal (streak ovaries) in these cases.

**46, XY Male**

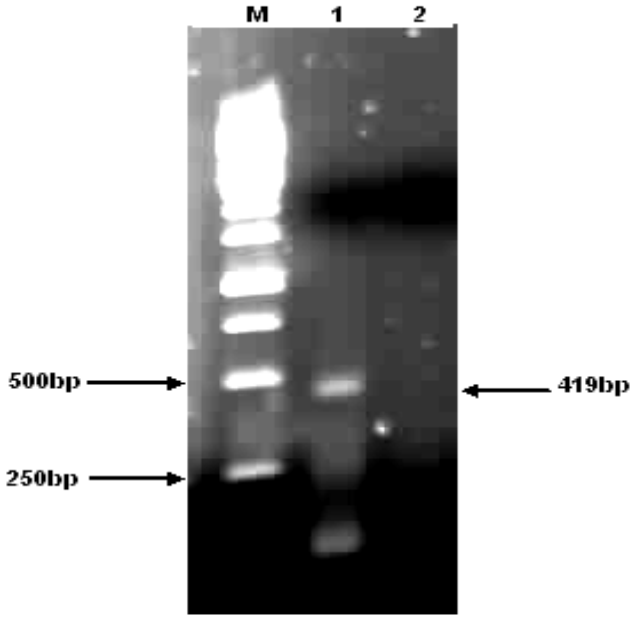


Fig. 4. PCR product of normal male after DNA digestion with *Hpa*II, selected as a control. Lanes 1 and 2 correspond to GAPDH segment and segment of exon 1 of the AR gene amplification, respectively. AR (lane 2) was not amplified after *Hpa*II digestion, despite adequate DNA amplification of GAPDH (lane 1) due to the presence of single X in 46, XY normal male. Lane M = molecular weight marker.

So TS was detected in 9 cases (6/9 45, XO). Out of these 30 affected cases 9 those were found to have TS karyotype, the mother's age was 32-45 years. Data shows that there is no correlation between mother's age and occurrence of TS (Table II). A mother can have a Turner child in 32 years as

well as 45 years of age.

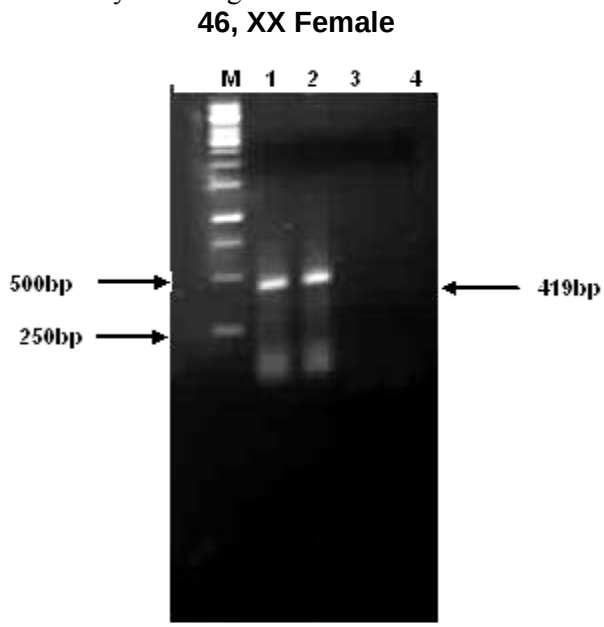


Fig. 5. PCR product of normal female after DNA digestion with *Hpa*II, selected as a control. Lanes 1 and 2 correspond to GAPDH segment and segment of exon 1 of the AR gene amplification, respectively. The 419 bp band (lane 2) correspond to amplification of AR gene segment protected from digestion by methylation due to the presence of two X in normal female. Lane M = molecular weight marker.

## DISCUSSION

TS is caused by the complete or partial loss of one sex chromosome in all or some cells. Complete absence of a second sex chromosome in females has a karyotype 45, XO. The karyotype 45, XO was first described by Ford and co-workers in 1959 and its occurrence was approximately 50% (Gravholt *et al*., 2000).

In 30 cases that were studied for TS, most prominent feature was short stature (~85%) among affected patients that were included, but other TS phenotype signs such as cubitus valgus, short neck, and widely separated nipples in short girls were also observed, but Turner karyotypes were found only in 9 cases. Similar study was carried out by Fergusson in 1993 and it was concluded that short stature is a symptom in 98% of cases of TS. Suri *et al.* in 1995 also found that short stature and primary amenorrhea were the presenting features in TS patients, regardless of karyotype. This indicated that some TS phenotype signs can be present in short girls, but these alterations are not specific for TS. It is, thus, important to accurately assess the incidence of TS in growth-retarded girls.

Females with the 45, XO karyotype are usually diagnosed earlier than those with mosaicism because their physical features tend to be more pronounced (Sybert and Mc-Cauley, 2004). In the present study the mean age of all females with 45, XO karyotype was 11 years while mosaicism with isochromosome was observed in one case with 18 years of age. The mean age of two other females was 16 years in which mosaic karyotype (45, XO/46, XX) was observed.

Traditional karyotyping is a routine diagnostic test used to study chromosomal aberrations such as TS but due to high cost of culturing and practical limitations of karyotyping this method is no more applicable as a routine screening test for high-risk groups, such as girls with short stature. The main drawback of the traditional karyotyping is the delay in obtaining the results. To prepare G-banded chromosomal spreads for karyotyping, a large amount of blood is required compared to molecular detection where amount of DNA in µg was required for a single test.

In this study molecular detection of 45, XO TS patients was based on the property that the second X chromosome is inactivated in blood and in most other tissues by methylation, in order to ensure expression of a single allele. At the same time, DNA methylation blocks DNA fragmentation by some restriction enzymes, such as *Hpa*II. X-chromosome methylation is absent in 46, XY or 45, XO subjects; therefore, after *Hpa*II digestion there is no amplification of genes spanning restriction sites on the X chromosome, such as the androgen receptor (AR) gene. This specific fragmentation also occurs in the AR gene at exon 1, a region containing two CCGG sequences. DNA digestion can prevent the amplification of this region during the PCR reaction. On the other hand, subjects with two X chromosomes (46, XX) will have only one chromosome digested while the other one remains as a template for a subsequent PCR amplification.

Pre-PCR *Hpa*II digestion was also used previously

**Table II.- Clinical and cytogenetic analysis in affected females.**

| Case no. | Age (Years) | Indication for diagnosis | Karyotypes | Mother's age |
|---|---|---|---|---|
| 1 | 10 | Short stature | 45, XO* | 35 |
| 2 | 11 | Short stature | 46, XX*** | 38 |
| 3 | 11 | Short stature | 46, XX | 30 |
| 4 | 12 | Short stature | 46, XX | 37 |
| 5 | 18 | Primary amenorrhea | 46, XX/46, Xi(Xq) ** | 32 |
| 6 | 11 | Short stature | 45, XO | 35 |
| 7 | 13 | Short stature | 46, XX | 42 |
| 8 | 9 | Short stature | 45, XO | 25 |
| 9 | 12 | Short stature | 46, XX | 45 |
| 10 | 8 | Lymphedema | 46, XX | 32 |
| 11 | 13 | Short stature | 46, XX | 35 |
| 12 | 8 | Short stature | 46, XX | 32 |
| 13 | 10 | Short stature | 45, XO | 35 |
| 14 | 11 | Short stature | 45, XO | 45 |
| 15 | 11.5 | Short stature | 46, XX | 32 |
| 16 | 3.5 | Abnormal features | 46, XX | 30 |
| 17 | 10 | Short stature | 46, XX | 35 |
| 18 | 13 | Short stature | 46, XX | 37 |
| 19 | 11 | Short stature | 46, XX | 35 |
| 20 | 13 | Short stature | 46, XO | 35 |
| 21 | 9 | Short stature | 46, XX | 32 |
| 22 | 18 | Primary amenorrhea | 46, XX/45, XO | 40 |
| 23 | 12 | Short stature | 46, XX | 35 |
| 24 | 11 | Short stature | 46, XX | 30 |
| 25 | 9.5 | Short stature | 46, XX | 36 |
| 26 | 20 | Gonadal dysgenesis | 46, XX | 43 |
| 27 | 16 | Primary menorrhea | 46, XX | 40 |
| 28 | 9months | lymphedema | 46, XX | 37 |
| 29 | 15 | Primary menorrhea | 46, XX/45, XO | 38 |
| 30 | 12 | Short stature | 46, XX | 37 |

*, monosomy; **, isochromosome; ***, normal female karyotype.

to detect TS in neonates (Rocha *et al*., 2005). In this study through PCR it was possible to complete the whole process in less than 12 hours; results reporting have become faster than the 5-7 days required for karyotyping. In the present study methylation sensitive *Hpa*II was used to detect methylation in X chromosome and ultimately number of X chromosomes.

Out of 9 TS patients 6 (66%) were found to have 45, XO karyotype. This detection by molecular method was also carried out by Longui *et al.* in 2002 on TS cases confirmed by karyotyping. Among 20 cases with 45, XO monosomy confirmed by karyotyping, mosaicism was found in 2 cases when molecular detection was carried out by same method (Longui *et al.,* 2002). Yorifuji *et al.* (1997) found that due to high sensitivity of this method to recognize 46, XX cell line this molecular method can also be applied to detect mosaicism in TS patients when 46, XX cell line is present in very small amount.

Presence of Y chromosomal material in Turner females can cause gonadoblastoma so detection of Y chromosomal material is required to prevent the occurrence of gonadoblastoma in short-stature girls. In the present study, Y chromosomal material was not detected because primers used were specific for HUMARA gene located on X chromosomes (Fig. 1), so in this method we can use primers specific for Y chromosome loci to detect Y chromosomal material in 45, XO females.

According to Gravholt *et al*. (1996) 45, XO

monosomy was usually detected in 50% of patients with TS, while mosaicism and X anomaly were detected in the other half while in this study, mosaicism and X anomaly were detected in 33% of the cases of diagnosed TS.

A limitation in the methodology of this present study is that it can only be applicable for the detection of 45, XO TS patients and does not have the potential to detect other chromosomal abnormalities usually observed in TS patients. But using other primers directed towards other methylated genes located in the short arm of X chromosome can potentially overcome these limitations. So it is concluded that molecular analysis of the X chromosome is a valuable alternative for cytogenetic analysis to diagnose TS in females with short stature.

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