Molecular diagnosis of Turner's syndrome

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Abstract

Turner's syndrome is a common disorder which occurs in around 1/3000 live births in girls. Diagnostic use of polymorphic DNA markers for the X chromosome could help to reduce the number of time consuming karyotype analyses needed. The M27 β probe maps on the X chromosome to Xcen-Xp11-22 and in 83% of female subjects detects heterozygosity with multiallelic polymorphism. In Southern blotting, a single X chromosome yields a single hybridisation band. In this study, genomic DNA was extracted from leucocytes of 49 patients with Turner's syndrome (karyotypes: 45,XO, n=29; 45,XO/46,XX, n=4; 46,Xi(Xq), n=1; 45,XO/46,Xi(Xq), n=4; 45,XO/46,Xr(X), n=4; 45,XO/46,XY, n=4; 46,XXp-, n=3), digested with *Eco*RI or *Hind*III, and analysed by Southern blotting. The molecular data for each patient were compared with DNA controls (homozygous 46,XX, heterozygous 46,XX and 46,XY DNA). A single band of reduced intensity compared to homozygous 46,XX control DNA was seen in 41 cases. Two hybridisation bands of different intensities were seen in four patients, in one of whom mosaicism was suspected on the basis of molecular analysis, despite a 45,XO karyotype. In four cases, Turner's syndrome failed to be detected: one 45,XO/46,XX mosaicism with only 4% of 45,XO cells and three distal Xp deletions. DNA analysis appears to be a useful and rapid tool in screening for Turner's syndrome and could be an alternative to cytogenetic analysis in diagnosing the disorder when severe growth retardation or delayed puberty are not accompanied by a Turner phenotype.

Turner's syndrome is one of the most frequently encountered chromosome abnormalities, its incidence being between 1/2500 and 1/10 000 live births among girls.^{1,2} In approximately 50% of cases, the karyotype anomaly is monosomy 45,XO, but a variety of other anomalies have been found including mosaicism, Xp or Xq deletion, and isochromy of the X long arm.¹⁻³

Recognition of the disease is important in view of current possibilities for therapy (bio-synthetic growth hormone treatment, for example),⁴ as is precise diagnosis of the chromosome anomalies responsible for the varying prognoses in terms of growth and fertility.⁵⁻⁷

Molecular biological techniques, using X chromosome polymorphisms and in situ

Table 1 Cytogenetic analysis in patients with Turner's syndrome.

Karyotype	No of cases	%
45,XO	29	59.2
45,XO/46,XX	4	8.2
46,Xi(Xq)	1	2
45,XO/46,Xi(Xq)	4	8.2
45,XO/46,Xr(X)	4	8.2
45,XO/46,XY	4	8.2
46,XXp-	3	6
Total	49	100

hybridisation, have proved to be useful tools in complementing the genetic approach to Turner's syndrome. These have helped to identify the parental origin of the X chromosome,^{8,9} the existence of cytogenetically unidentified mosaicism,^{8,9} and the X regions involved in phenotypic manifestations of the syndrome.¹⁰

The aim of this work was to determine whether DNA analysis in cases of Turner's syndrome, using a genomic probe for the X chromosome for which restriction polymorphism is particularly frequent, might constitute a valid diagnostic test and to evaluate its usefulness in conjunction with conventional karyotype investigation.

Subjects

CONTROL SUBJECTS

Ninety-seven Caucasian control subjects were investigated, 75 women and 22 men.

PATIENTS

Analyses were done on 49 girls with Turner's syndrome (table 1). Diagnosis of the syndrome was established at birth or within the first year in 19 patients (15 cases of 45,XO monosomy and three of the four 45,XO/46,XY mosaicism) in view of dysmorphism, malformations, or sexual ambiguity, and after the age of 6 years in the remaining patients because of growth retardation. Ages ranged from 1 to 19 years at the time of the molecular investigation (mean 13 years 2 months (SD 4 years 2 months)).

FAMILY STUDIES (TABLE 2)

Family studies were carried out on 12 families. Maternal and paternal DNA was available for nine families and only maternal DNA was available for the other three families.

Among the 12 mothers, four (table 2, families 8, 9, 11, and 12) were suspected of having Turner's syndrome because of clinical signs of hypogonadism (family 8, oligomenorrhoea, secondary sterility, early menopause) or short

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Received 24 September 1991.

Revised version accepted 7 February 1992.

Table 2 Data from family studies.

Family	Daughter			Final height (cm)	Mother	
	Karyotype	DNA analysis*	Origin of normal X		Karyotype	DNA analysis*
(1) Am	XO	1 band	Maternal	158	/	2 bands
(2) Ba	XO	1 band	Maternal	161	/	2 bands
(3) Cl	XO	1 band	Paternal	170	/	2 bands
(4) Du	XO	1 band	Maternal	165	/	2 bands
(5) Fe	XO	1 band	Paternal	153	/	2 bands
(6) For	XO	1 band	Maternal	167	/	2 bands
(7) Lec	XO	1 band	Maternal	156	/	2 bands
(8) Te	1st XO 2nd 84%XO/16%Xr(X)	2 ≠ bands	Paternal	149.5†	52%XO/48%Xr(X)	1 dble band 2 ≠ bands
(9) Ah	80%XO/20%XX	2 ≠ bands	Maternal	150	/	2 bands
(10) Le	79%XO/21%XX	2 ≠ bands	Paternal	173	/	2 bands
(11) Gr	60%XO/40%Xr(X)	1 band	Maternal	151	/	2 bands
(12) Pr	76%XO/24%Xr(X)	1 band	Paternal	149.5	XX	2 bands

* 1 band = one band of 50% reduced intensity compared with homozygous 46,XX DNA. 2 ≠ bands = two bands of different intensities. 1 dble band = restriction profile identical to 46,XX homozygous DNA. 2 bands = restriction profile identical to 46,XX heterozygous DNA.

† Gonadal disease.

stature (families 8, 9, 11, and 12). For the other families, the study was made systematically.

Methods

KARYOTYPE

Karyotype analysis was performed according to a method previously described.¹¹ Between four and 50 lymphocytes were examined in each case (mean 23 cells).

PREPARATION OF GENOMIC DNA

Miller's technique¹² was used, with slight modifications.¹³ Leucocyte DNA was isolated from 10 ml blood samples collected in EDTA and stored at -80°C until extraction of the DNA. Samples were then heated at 37°C and washed twice with 40 ml 0.02 mol/l Tris-HCl, pH 7.5, 0.005 mol/l MgCl₂. The lysed cells were resuspended in 3.5 ml 0.02 mol/l Tris-HCl, pH 7.5, 0.4 mol/l NaCl, 0.002 mol/l EDTA, and then incubated overnight at 56°C with 0.2 ml 10% SDS and 80 µl proteinase K (10 mg/ml). Proteins were precipitated by addition of 1 ml saturated NaCl, shaking, and centrifuging. Two volumes of absolute ethanol were added to the supernatant at room temperature and the resulting precipitated DNA was resuspended in 0.2 ml 0.01 mol/l Tris HCl, pH 7.5, 0.001 mol/l EDTA. Spectrometry at 260 and 280 nm was performed for DNA quantification.

DNA PROBE

The M27β probe, a 2.3 kb anonymous single copy DNA fragment subcloned in pUC 9, was kindly provided by Dr I Craig.¹⁴ This was isolated from a genomic library enriched for human X chromosomal material.¹⁵ It maps on the short arm of the X chromosome to Xcen-p11.22 in a variable number tandem repeat (VNTR) region. Transmission of polymorphism is mendelian. The informativity of M27β has been tested with numerous restriction enzymes (*Bgl*I, *Bgl*II, *Eco*RI, *Hind*III, *Msp*I, *Pst*I, *Pvu*II, and *Taq*I). The autosomal IGFII probe mapping on chromosome 11p1.5¹⁶ was used as a control of DNA deposit.

SOUTHERN BLOT ANALYSIS

DNA (10 µg) was digested with 100 units *Eco*RI (New England Biolabs Inc, USA) or *Hind*III (Appligene, Illkirch, France) and the digested DNA submitted to 0.7% agarose gel electrophoresis. DNA quantification was systematically checked by ethidium bromide staining. Transfer and hybridisation were performed according to Chomczynski and Qasbas.¹⁷ The probe was labelled with α³²P-ATP (Amersham, England) by random priming (Kit Amersham, England. Specific activity = 1.5 to 2 × 10⁹ cpm/µg DNA). The concentration of probe in the hybridisation medium was approximately 10⁷ cpm/ml.

DENSITY MEASUREMENTS

Hybridisation signals were estimated from the autoradiographs in comparison with two female reference DNAs (a homozygous 46,XX and a heterozygous 46,XX) and a male reference 46,XY DNA. Hybridisation intensities of polymorphic alleles were quantified by densitometry using a GS 300 Scanning Densitometer and GS-370 1D electrophoresis data system (Hoefer Scientific Instruments, San Francisco, USA). A single band of reduced intensity (50% of the homozygous 46,XX control DNA) reflects a single X chromosome. DNA deposit quantification was checked by a second hybridisation with an autosomal sequence, the IGFII probe. Two bands of different intensities reflect mosaicism.

Results

RESTRICTION PROFILES IN THE CONTROL SUBJECTS

Restriction polymorphisms were investigated with the enzymes *Bgl*I, *Bgl*II, *Eco*RI, *Hind*III, *Msp*I, *Pst*I, *Pvu*II, and *Taq*I in 75 women. In 33 cases, between two and eight different restriction enzymes were used. When polymorphism was found for one enzyme in a given subject, it was also evident for the other enzymes tested. With *Eco*RI and *Hind*III, the restriction fragments varied between 3.5 and 7 kb.

The degree of informativeness in the control Caucasian population was 83% (multiallelic

Table 3 Restriction profiles in patients with Turner's syndrome.

Karyotype	No	Single 50% hybridisation band	2 bands of different intensities	Normal 46,XX profile
45,XO	29	28	1	
45,XO/46,XX	4		3	1
46,Xi(Xq)	1	1		
45,XO/46,Xi(Xq)	4	4		
45,XO/46,Xr(X)	4	4		
45,XO/46,XY	4	4		
46,XXp-	3			3
Total	49	41	4	4
			Positive 92%	False negative 8%

Figure 1 Restriction profiles of patients with Turner's syndrome; 10 µg of leucocyte DNA restricted with HindIII. Lanes 1-5: 45,XO, lanes 6-8: 45,XO/46,Xr(X), lanes 9-11: DNA controls = 9: 46,XY; 10: heterozygous 46,XX; 11: homozygous 46,XX. Panel A: hybridisation with the X probe M27β. Panel B: hybridisation with the autosomal IGFII probe.

Figure 2 Restriction profiles of Turner's syndrome patients with mosaicism; 10 µg of DNA restricted with EcoRI. Te D and Te M (daughter and mother): 45,XO/46,Xr(X); Ah, La, Le: 45,XO/46,XX. DNA controls: 46,XY, 46,XX.

restriction polymorphism in 62 of the 75 women tested). A single hybridisation band was found in all 22 of the men tested.

RESTRICTION PROFILES IN THE PATIENTS WITH TURNER'S SYNDROME (TABLE 3)

In 41 of the 49 cases, a single hybridisation band was found of 50% reduced intensity compared with the homozygous reference 46,XX DNA (fig 1). The hybridisation band with the autosomal probe IGFII confirmed that DNA in each lane was nearly equivalent.

In four cases there were two bands of different intensities (fig 2). In three of these (fig 2, patients Ah, La, Le), cytogenetic examination showed 45,XO/46,XX mosaicism. There were 20%, 50%, and 21% of cells, respectively, carrying 46,XX, which agreed with the Southern blotting data (fig 3A, B, C, D). In the fourth patient (fig 2, patient Te D), who had undergone spontaneous puberty, cytogenetic analysis (15 cells examined) showed 45,XO monosomy. With DNA analysis, the diagnosis could be refined, since the two bands of very different intensities indicated mosaicism with a particularly small proportion of 46,XX cells. Karyotype analysis was done on the basis of these findings and showed 45,XO/46,Xr(X) mosaicism with a 46,Xr(X) population of 16%, which is slightly higher than that suspected from the molecular data (fig 3A, E).

In four cases, DNA analysis failed to confirm the diagnosis of Turner's syndrome, the Southern blot profile being identical to the 46,XX control population (fig 4). Two had the single band with the same intensity as the homozygous 46,XX control DNA: one of these proved to involve 45,XO/46,XX mosaicism with only 4% of the abnormal 45,XO cells, which could be identified only after a second karyotype analysis had been done using 50 cells; in the other patient, there was a distal Xp deletion. The remaining two patients had restriction profiles identical to the heterozygous 46,XX DNA control and, here again, there were Xp deletions.

FAMILY STUDIES

In one girl (table 2, family 8) who had undergone spontaneous puberty, diagnosis of 45,XO monosomy was corrected to 45,XO/46,Xr(X) mosaicism on the basis of molecular analysis. Turner's syndrome was suspected in the mother in view of short stature (149.5 cm), secondary sterility, and premature menopause (at the age of 35) and confirmed first by molecular analysis (two bands of different intensities, fig 2, Te M) and then by cytogenetic analysis (45,XO/46,Xr(X) mosaicism). As for the daughter, the 46,Xr(X) cell population was slightly higher than that suspected from molecular data (fig 3A). Molecular analysis showed that the abnormal allele was of maternal origin (figs 2 and 3E).

In the three families (table 2, families 9, 11, and 12) where the mother had short stature, the mother's restriction profiles were normal (identical to 46,XX heterozygous DNA control). Moreover, the multiallelic polymorphism of M27β allowed the determination of the parental origin of the normal X chromosome with the use of only one DNA marker (table 2, fig 5). We showed that the majority

A

Patients	Cytogenetic analysis	Theoretical density ratio superior to inferior bands	Measured density ratio superior to inferior bands
Ah	80% XO/20% XX	0.83/0.17	0.80/0.20
La	50% XO/50% XX	0.33/0.66	0.42/0.58
Le	79% XO/21% XX	0.17/0.83	0.09/0.91
Te D	84% XO/16% Xr(X)	0.86/0.14	0.94/0.06
Te M	52% XO/48% Xr(X)	0.33/0.67	0.19/0.81

D = daughter, M = mother

Figure 3 Density measurement data from Turner's syndrome patients with mosaicism. (A) Comparison of the degree of mosaicism by chromosome and DNA analysis. (B, C, D, E) Densitometric profiles of the four Turner mosaics.

of 45,XO had a maternal X chromosome, whereas mosaics had a normal X chromosome of either maternal or paternal origin.

Figure 4 Molecular analysis in Turner's syndrome patients with false negative molecular analysis; 10 µg of DNA restricted with EcoRI. Lanes 1-2: 46,XXp-, lane 3: 4% 45,XO/96% 46,XX; lanes 4-6: DNA controls=4: 46,XY; 5: heterozygous 46,XX; 6: homozygous 46,XX.

Discussion

If Southern blot analysis were to be applicable as a routine diagnostic tool for Turner's syndrome, a single and highly informative probe would need to be used. The M27β probe was 83% informative in the control Caucasian population with the restriction enzymes tested (*Bgl*I, *Bgl*II, *Eco*RI, *Hind*III, *Msp*I, *Pst*I, *Pvu*II, *Taq*I), which is well above the percentage seen for probes previously used in studying Turner's syndrome.⁸

With comparison with control DNA (homozygous and heterozygous women and man), the sensitivity of the test was estimated to be 92% (8% false negatives) and its specificity to be 100%. Furthermore, DNA analysis is usually found to be in agreement with the chromosome data.^{18,19}

The advantages of DNA analysis over cytogenetic studies should be emphasised. First, molecular analysis is rapid (results within four days) and several samples can be treated in parallel. In view of its simplicity, the test would be applicable to broader screening of larger numbers of patients. Short stature is a symptom in 98% of Turner's syndrome, but dysmorphism is often absent.^{20,21} However, there are many cases of severe growth retardation which appear inexplicable (lack of endocrine disorder or chronic illness) and for which there appears to be no clinical indication for karyotype examination (dysmorphism or somatic abnormality). Here, Southern blot analysis could be an alternative and, if Turner's syndrome is suspected on the basis of DNA data, the karyotype would confirm diagnosis and identify precisely the abnormality concerned. The two types of test are therefore complementary.

With cytogenetic analysis, the possibility of mosaicism cannot be excluded without large numbers of mitoses being examined.²² Furthermore, in vitro cell selection may affect the percentage of cells in a given karyotype.^{23,24} With leucocyte DNA analysis, larger numbers of cells can be studied and, to this extent, greater sensitivity is achieved in recognising mosaicism.^{8,9} In the cases of mosaicism, partial digestion could be ruled out because restriction profiles of the parents confirmed the size of the two bands in the daughter (fig 2). Many authors^{19,25} have postulated that 45,XO monosomy is lethal (99% of these result in spontaneous abortion) and that most surviving 45,XO subjects are in fact mosaics undetected by cytogenetic tests. Use of this particularly informative DNA marker (M27β) would provide a simple test to detect hidden mosaicism. In this study, just one hidden mosaic was detected but only blood leucocytes were examined and it is possible that mosaicism existed in other tissues.

Further advantages of molecular analysis include the possibility of examining non-dividing tissues, whereas cytogenetic analysis requires actively dividing cells, so its application to investigation of spontaneous abortions is possible.

There are, nevertheless, limitations to DNA analysis. Use of a proximal probe means that

Figure 5 Molecular analysis of the families; 10 µg of DNA restricted with *EcoRI*. D= daughter, F=father, M=mother. DNA controls: 46,XY; heterozygous 46,XX; homozygous 46,XX.

distal p and q deletions fail to be detected, which was the case in most of the false negatives obtained. It would therefore be necessary, when restriction profiles appear normal but there is clinical suspicion of Turner's syndrome, to complement the test with p and/or q specific distal probes.

45,XO/46,Xr(X) mosaicism may also be deceptive. The expected profile comprises two bands of different intensities, but in the four cases cytogenetically recognised as 45,XO/46,Xr(X) mosaicism, a single hybridisation band was obtained. There are two possible explanations for this non-recognition of the ring chromosome. First, the probe may fail to recognise the X ring involved. It is known from cytogenetic data that the ring X can exist in two forms, a small and a large ring depending on the chromosomal material deleted.^{26,27}

Nevertheless, in this study, 45,XO/46,Xr(X) mosaicism was recognised in one proband and her mother, although the hybridisation signals were weaker than expected from cytogenetic analysis (fig 3A, E). The second explanation involves the frequent difficulty in distinguishing a ring X from a Y ring by cytogenetic analysis. Since virilisation does not always occur in cases of r(Y) mosaicism²⁸ and, in addition, there is a high risk of gonadoblastoma,^{28,29} it would be important in these four cases that any possible XO/Xr(Y) be identified using Y specific probes.³⁰ Here again, molecular analysis would give complementary information to cytogenetic analysis.

Finally, on the basis of the family studies, Turner's syndrome was recognised in one mother and it was confirmed that a ring X chromosome, which is characteristically unstable,^{26,31} had been transmitted to the daughter. Moreover, with RFLPs of M27β, the parental origin of the X chromosome abnormality can be determined with only one DNA marker.

We are greatly indebted to Dr Ian Craig for his generous gift of the M27β probe. This work was supported in part by the Association Française contre les Myopathies and in part by Université Paris VI.

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