Cytogenetic and molecular findings in patients with Turner's syndrome stigmata

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Abstract

Cytogenetic and DNA analysis in 12 people with stigmata of Turner's syndrome was carried out. Cytogenetic analysis of these patients showed two subjects with 46,X,i(Xq) karyotypes, one with 45,X/46,X,i(Xq), one with 46,X,t(X;Y), and eight with 45,X/46,X,mar. Molecular analysis of DNA samples was performed in nine out of 12 patients with marker chromosomes. PCR analysis with oligoprimers specific for SRY, DYZ1, or DYZ3 loci identified Y chromosome material in five patients in the latter group. The X chromosome origin of the marker chromosome was proved by FISH technique with biotin labelled pericentromeric X chromosome specific probe in four other patients. These results show that patients with a number of Turner's syndrome stigmata usually do not have a typical XO karyotype but have some structural chromosomal aberrations involving the X or Y chromosomes. Combined application of cytogenetic, molecular cytogenetic (FISH), and PCR techniques significantly improves the precision of marker chromosome identification and thus might be of practical importance for the proper management and treatment of affected patients.

Peculiarities of pathological manifestations of different karyotypes bearing structural abnormalities of the X or Y chromosomes in patients with Turner's syndrome stigmata, as well as feasible genetic mechanisms of sex determination and differentiation abnormalities in these subjects, are briefly discussed.

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developmental cytogenetics has already been quite thoroughly elaborated in mice² but still remains in its infancy in humans.

Complex comparative clinical, cytogenetic, and molecular analysis is also important to determine the nature of marker chromosomes because of the high risk of malignancy in dysgenetic gonads owing to the presence of Y chromosome material.^{3,4}

Mosaic karyotypes with small marker chromosomes represent a difficult task for cytogenetic analysis by routine classical methods. However, the combination of cytogenetic and molecular techniques enables this problem to be solved quite efficiently and the origin of markers in patients with Turner's syndrome to be established.⁴⁻⁶

We have used such a complex approach to examine 12 patients with stigmata of Turner's syndrome for precise karyotyping, identification of the origin of marker chromosomes, and determination of the Y material in these subjects.

Materials and methods

PATIENTS

All 12 patients reported here are unrelated females with ages ranging from 7 to 32 years. Patient 8 has been previously reported.⁶ The main reasons for their karyotyping were short stature, hypoplasia of the external genitalia, and delayed puberty. Nine of them were subjected to ultrasonographic examination. Laparoscopy with subsequent gonadal biopsy or gonadectomy and histological study of the gonads was carried out in four cases. Clinical details of each subject are presented in table 1.

KARYOTYPING

Karyotype analysis was performed by standard cytogenetic techniques using metaphase chromosome preparations from PHA stimulated peripheral blood lymphocytes. Fluorochrome Hoechst 33258 contrastained with actinomycin D or replicative banding based on BrdU incorporation was used for cytogenetic analysis. The number of metaphases scored for each patient, the percentage of cell clones in mosaics, and the replication status of marker chromosomes are presented in table 2.

FISH TECHNIQUE

The following biotin labelled pericentromeric specific DNA probes were used: pYAM10-40 for the X chromosome⁷ and Y97⁸ and pHY2.1

According to recent reports only about half of all Turner's syndrome patients are really monosomic for the whole X chromosome, while the other half is represented by a heterogeneous group with different structural abnormalities of the sex chromosomes.¹ Most of them are confined to structural rearrangements of the X chromosome. The other group of these patients have a mosaic karyotype with the second cell lines carrying numerical or structural sex chromosomes anomalies.¹ Detailed clinical and cytogenetic analysis of these patients can provide new information on the developmental effects of different chromosomal segments and their participation in normal and abnormal development. De-

Table 1 Patient data

	Patient											
	1	2	3	4	5	6	7	8	9	10	11	12
Age (y)	22	32	17	26	20	7	14	14	18	22	19	22
Clinical features												
Short stature	+	+	+	+	+	+	+	+	+	+	+	+
Epicanthic folds		+		+		+	+				+	+
Arched palate	+		+	+	+	+	+		+		+	+
Micrognathia		+		+		+	+				+	+
Short neck	+		+	+	+		+	+	+	+	+	+
Low hairline				+					+		+	+
Shield chest			+	+	+			+	+	+		
Cubitus valgus	+		+	+	+	+	+	+				+
Thyroid gland hypertrophy		+					+	+				
Mental retardation						+						
External genitalia												
Normal female								+		+		
Hypoplasia	+	+	+	+	+		+		+		+	+
Gonads												
Not detected			+	+	+		+			L		L
Rudimentary								+			+	R
Streak	+								+	R		
Uterus												
Hypoplasia							+	+				+
Streak				+	+				+	+		
Double horned												+
Histology												
Degenerative follicles								+				
Leydig cells											+	+
Wolffian ducts									+			

for the Y chromosome.⁹ The hybridisation procedure was as described elsewhere.¹⁰ Hybridisation signals were scored both in metaphases and in interphase nuclei (500 cells in total).

PCR ANALYSIS

DNA samples extracted from peripheral blood lymphocytes by means of conventional proteinase K method were used for PCR with specific oligoprimers corresponding to the DYZ1¹¹ and DYZ3¹² loci of the Y chromosome. Analysis of the SRY locus was performed by nested PCR with XES2 and XES7¹³ as external primers, and SRY1 and SRY2¹⁴ as internal ones.

Results

The clinical details of the 12 patients analysed are shown in table 1. All of them showed clear cut short stature and hypoplasia of the genitalia without genital ambiguity. Rudimentary or streak gonads and uteri were registered in nine

cases but ultrasonographic examination could not be done in cases 2, 4, and 6. Other Turner's syndrome stigmata included short neck (cases 1, 3, 4, 5, 7, 8, 9, 10, 11, 12), low hairline (cases 5, 9, 11, 12), shield chest (cases 3, 4, 5, 8, 9, 10), high palate (cases 1, 3, 4, 6, 7, 9, 11, 12), some minor skeletal malformations such as cubitus valgus (cases 1, 3, 4, 5, 6, 7, 8, 12), and micrognathia (cases 2, 4, 6, 7, 11). Some of the patients had other clinical stigmata and pathological traits not confined to Turner's syndrome, such as thyroid gland hypertrophy (cases 2, 7, 8) and mental retardation (case 6). Thus Turner's syndrome stigmata including short stature and underdeveloped, rudimentary, or absent gonads were typical for each of our patients. None of them, however, had other classical Turner's syndrome traits such as webbed neck, congenital heart defects, generalised lymphoedema, aortic coarctation, etc.

Chromosomal analysis of QFH/AcD banded chromosomes (data not shown) ascertained an abnormal karyotype in each of the 12 patients under study with eight of them carrying one rearranged X chromosome (cases 1–8) and five

Table 2 Combined results of cytogenetic and molecular studies of the patients

Patient	Cytogenetic			Molecular (PCR)			FISH with probe		
	Karyotype	Cell No*	Replication of marker	Cell No†	SRY	DYZ3	DYZ1	pYAM10–40	Y97
1	46,X,idi(Xq)	11	Late	15		ND		+	ND
2	46,X,i(Xq)	11	Late	20		ND		+	ND
3	45,X/46,X,i(Xq) (50%)	20	Late	30		ND		+	ND
4	45,X/46,X,r(X) (10%)	30	Early	50	–	–	–	+	ND
5	45,X/46,X,r(X) (15%)	50	Early	50	–	–	–	+	ND
6	45,X/46,X,dicr(X) (30%)/47,X,f(X),f(X)(10%)	80	Early	60	–	–	–	+	ND
7	45,X/46,X,r(X) (10%)/46,X,dic(X) (5%)	110	ND		–	–	–	+	ND
8	46,X,t(X;Y)	11	Early	100	–	–	+	+	ND
9	45,X/46,X,der(Y) (50%)	40	Early	40	+	+	–	+	–
10	45,X/46,X,idi(Xp) (40%)	50	Early	40	+	+	–	+	ND
11	45,X/46,X,idi(Xp) (15%)	100	ND		+	+	–	ND	ND
12	45,X/46,X,Yq- (3%)	100	ND		+	+	–	ND	ND

ND = not done.
* For all mosaics (excluding case 12) total cell number analysed was 500 (metaphase plates + interphase nuclei after FISH).
† Metaphase plates with marker.

Figure 1 Fluorescent in situ hybridisation of biotin labelled probes pYAM10-40(B), Y97 (C) and simultaneously pYAM10-40 and pHY2.1 (A) to metaphase chromosomes from (A) patient 8, (B) patient 7, and (C) patient 10 (see text and table 2 for details). V indicates the marker chromosomes, W indicates the normal X chromosomes.

of them having Y chromosome material (cases 8–12). The karyotypes of these patients as well as the results of corresponding FISH and molecular studies are summarised in table 2.

A single blood lymphocyte line with a 46,X,der(X) karyotype was found in three patients (cases 1, 2, 8) with two of them bearing Xq rearrangements, i(Xq) (case 1) and idic(Xq) (case 2), as proved by FISH analysis with alphoid pericentromeric DNA probe pYAM10-40. In the remaining single cell line case (case 8) a Xp22.3;Yq11 translocation was suspected and later proved by FISH with

pYAM10-40 and pHY2.1 DNA probes (fig 1A) as well as by PCR analysis (fig 2, top).

The presence of at least one more chromosomally abnormal line was registered in nine cases (cases 3, 4, 5, 6, 7, 9, 10, 11, 12) and two additional lymphocyte cell lines were identified in cases 6 and 7. The proportions of different cell lines in mosaics scored both in metaphase plates and in interphase nuclei after in situ hybridisation with relevant DNA probes varied from 3% (case 11) to 50% (case 5) (table 2).

FISH and molecular analyses were undertaken for identification of marker chromosomes in all nine mosaics in whom routine cytogenetic methods had failed to define the origin of small marker fragments. The marker chromosomes in both cases 4 and 5 (data not shown) were small rings bearing X chromosome centromeric regions with no indication of Y chromosome material (table 2).

A dicentric X chromosome derivative predominantly of a ring shape was identified in 30% of cells in case 6. Two rod shape markers with only one centromere were found in a small proportion of metaphases in the same subject (data not shown).

Three cell lines 45,X/46,X,r(X)/46,X,dic(X), in proportions 85%, 15%, and 5% respectively, were found in case 7 (fig 1B).

Cases 8 to 12 were proved to bear Y chromosome material (table 2). One of them (case 8) was non-mosaic, as was reported earlier, while the remaining ones were mosaics with different ratios of Y chromosome fragment cell lines (table 2).

PCR analysis with oligoprimer systems specific for different parts of the Y chromosome was undertaken to identify the constitution of Y fragments in the karyotypes of the previous group of patients (table 2). Presence of the DYZ1 locus was found only in case 8 (fig 2, top). Locus DYZ3 corresponding to the pericentromeric region of the Y chromosome (fig 2, top) as well as the SRY gene (Yp11.3) (fig 2, bottom) were ascertained for the marker chromosomes in cases 9, 10, 11, and 12.

Patients 9 to 12 were positive for the centromeric heterochromatin region, and two of them (case 10, fig 1C; case 11, data not shown) were identified as dic(Yp) by FISH with the Y97 DNA probe.

Only three metaphases with a small Y chromosome specific centromeric fragment were found in 100 metaphases of case 12. The presence of this cell line in about 5% of lymphocyte cells has been proven by FISH studies of interphase cells as well as by PCR analysis of DNA samples of this subject (fig 2).

Replication pattern of marker chromosomes was undertaken for 10 patients. Both i(Xq) (cases 2 and 3) and idic(Xq) (case 1) were late replicating while the X;Y translocation product in case 8 showed a preferential early pattern of replication (data not shown) in more than 90% of metaphase cells scored (table 2). The ring (X)s (cases 4 and 5) including dicr(X) (patient 6) were found to be early replicating after BrdU staining (fig 3) in all cells in which they were present (table 2).

Figure 2 Y chromosome specific sequences amplified by PCR with oligoprimers corresponding to DYZ3 and DYZ1 (top) (170 and 150 bp respectively) and SRY (bottom) (600 bp and nested PCR 330 bp). PCR products of DYZ3 and DYZ1 (top) and both fragments of SRY (bottom) were electrophoresed together. Lane M: DNA digested with PstI (size marker); lanes 1–7: peripheral blood DNA from control male (lane 1), control female (lane 2), and patients 8 (lane 3), 9 (lane 4), 10 (lane 5), 11 (lane 6), and 12 (lane 7).

Figure 3 Replication patterns of the X and ring (X)s from PHA stimulated lymphocytes of patient 4 (A), 5 (B), and 6 (C). Both the ring (V) and the normal X chromosome (W) are early replicating after BrdU incorporation for the last six hours of culturing following FPG staining.

Early replicating Y chromosome marker derivatives were determined in two patients (cases 9 and 10) (data not shown).

This study has not yet been performed in cases 7, 11, and 12 (table 2).

Discussion

Two basic hypotheses on the manifestation of Turner's syndrome can be considered; first, the existence of two X chromosomes for the survival of early human XO conceptuses,¹¹⁵¹⁶ and, second, haploinsufficiency or imbalance of some gene products in non-activated homologous regions of the X and Y chromosomes.^{17–20}

It is well known that over 90% of all XO conceptuses are eliminated during early prenatal development and chromosomal mosaicism, involving both X and Y chromosomes, has often been found in Turner's syndrome patients postnatally.⁵¹⁵²¹ According to the present findings nine of 12 patients studied were found to be mosaics with at least one chro-

mosomally abnormal cell line bearing X or Y chromosome fragments. The presence of a second aberrant cell line was registered in three cases only, with two of them being represented by an abnormal i(X) chromosome and one by an Xp;Yq translocation product. Thus, a definite proportion of chromosomally abnormal cells in each patient studied contained a double dose of chromosomal material of both gonosomes. In view of the present hypothesis the existence of genetic material of two identical (X/X) or heterologous (X/Y) chromosomes could be sufficient to allow survival of the relevant conceptuses during early embryogenesis when genetic activity of some homologous loci on both gonosomes are required for normal development.¹⁵²¹ Our data are thus in line with the hypothesis of proven or cryptic chromosomal mosaicism as indispensable for successful survival of XO conceptuses.

None of our patients showed a classical pattern of Turner's syndrome and some of them had pathological traits of other inherited disorders. Some of these findings could be at-

tributed to haploinsufficiency of the gene products stemming from the imbalance of the genes in non-inactivated portions of both gonosomes, such as RPS4X/RPS4Y¹⁷ and ZFX/ZFY.¹⁸ Haploinsufficiency of some other gene products might also contribute to the phenotypic manifestations in Turner's syndrome patients with X and Y chromosome rearrangements.²² Imbalance of these particular genes might be predicted in all our patients lacking portions of Xp22.3 or Yp11.2.

Terminal Xp deletion resulted in abnormal development in patient 8 with an Xp22.3;Yq11 translocation. Most probably the active state of the rearranged chromosome resulted in Turner's syndrome in this particular case.⁶

Imbalance of the above mentioned genes as well as the XIST locus might account for at least some Turner's syndrome stigmata^{23,24} in the patients with early replicating marker chromosomes bearing relevant fragments (cases 4, 5, 6, 9, and 10 in this report).

It should be noted that a cytogenetic judgement on the structure and replication status of small marker chromosomes is far from being sufficient to prove the presence and activity of some relevant genes (see above).

The early replicating small ring (X)s lacking the XIST gene are usually associated with a severe phenotype.^{19,23,24} The presence of the X inactivation centre (XIST) in ring (X)s in patients 4 and 5 with a mild Turner phenotype and its absence in dicr(X) in patient 6 with mental retardation in addition to Turner's syndrome stigmata might be possible. It should be mentioned, however, that failure of X chromosome inactivation, as might be inferred from direct XIST gene expression studies in X chromosome rearrangements, does not explain the severe phenotype of affected patients.²⁵

Conspicuous clinical polymorphism in each of our patients justifies more precise identification of chromosome breakpoints for better understanding of developmental effects caused by the structural chromosomal imbalance.

Sizes and frequencies of rearranged marker chromosomes might also contribute to the phenotypic diversity.²³ The latter might also be attributed to substantial variability of each genetically abnormal cell line in different tissues.^{15,26} Analysis of mosaic cell lines in blood lymphocytes is hardly applicable for the proper estimation of the cell proportion with an abnormal karyotype in the other tissues. This could be the explanation in case 12 with a very small proportion of Yp fragment bearing cells (3%) in blood but with definite Leydig cells in her rudimentary gonads.

The basic sex determining factor responsible for switching on male development is known as the SRY gene, located in Yp11.2 very close to the pseudoautosomal region of this chromosome.²⁷ According to recent data, the regulatory gene responsible for switching to the female pathway of sex differentiation is mapped to Xp21.1–21.3.^{28–30} Some other autosomal genes are also suspected to be involved in phenotypic sex differentiation as well.^{31–33}

All our patients were female on external examination. Underdevelopment of the ex-

ternal genitalia and gonads in all subjects with X chromosome aberrations (cases 1–7) might be attributed to the same still unknown mechanisms which are responsible for primordial germ cell elimination and development of streak gonads in the patients lacking one X chromosome. It is possible that early replication of Xq chromosomal fragments bearing RPS4X, ZFX, and XIST genes in the absence of an ovarian differentiation gene mapped to Xp21 could not provide normal development of female genitalia in these cases.

A female phenotype in all our subjects bearing Y material is a more difficult task for adequate interpretation. No SRY gene was found in case 8 and thus her female phenotype might be attributed to the absence of a regulatory SRY gene product. Both SRY fragments screened by PCR were discovered in four other female patients with cytogenetically and molecularly proven Y chromosome cell lines. Typical Leydig cells and rudimentary seminiferous tubules were found in histological sections of gonads in three of them (cases 9, 11, and 12). The reason for the femaleness in these patients with Y chromosome cells remains completely obscure. Whether their phenotype could be attributed to some inefficiency of SRY as a transcriptional repressor or whether its activity has been disrupted by two doses of active ovarian differentiation genes in Xp21.1–21.3^{28–30} or by the proportion of Yp positive cells in different tissues needs to be clarified.

The results of the present study provide evidence for the importance of complex cytogenetic and molecular approaches in the identification of marker chromosomes and precise analysis of X and Y chromosome aberrations. Each of these methods has its own advantages and drawbacks which should be carefully considered before application. Classical cytogenetic methods provide more general information on karyotype structure and chromosomal rearrangements but are hardly applicable for marker chromosome identification or exact breakpoint location. The former might be much more easily attained with FISH, while the latter goal might be readily achieved by DNA analysis, which is more rapid, precise, and efficient. Meanwhile both FISH and DNA analysis have their own limitations and rely on the knowledge of karyotype structure and rearrangements for precise identification.

It should be pointed out, however, that application of the FISH technique in mosaic cases with a small number of abnormal cell lines (case 12) is not of great diagnostic value as the specific labelling signals are very difficult to discriminate from the background level. The same is true for the SRY gene analysis by standard PCR in mosaics with a very low proportion of Y positive clones (too faint specific amplification bands in cases 11 and 12) (fig 2, bottom). Application of PCR with nested oligoprimers has helped to solve this problem.

We are convinced by the present results, as well as by data of other authors,^{4,21,34} that only the combined application of cytogenetic and molecular techniques can enable adequate chromosomal analysis in patients with marker

chromosomes or with complex chromosome rearrangements.

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