Genetic analysis of Turner syndrome in Tunisian patients: from diagnosis to management

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Background/aim
Turner syndrome (TS) is a rare sex chromosome abnormality in women, occurring in approximately one in 2500 live births, associated with a wide range of clinical stigmata of which short stature, ovarian dysgenesis, and dysmorphic features are the most frequent. Morbidity and mortality are clearly increased compared with the general population, and the average age at diagnosis is quite delayed. Even if the majority of females with TS have a non-mosaic 45,X karyotype, several karyotype variations exist, including short or long arm deletion, ring X isochromosome of the long arm, and 45,X 46,XX mosaicism. This explains the large phenotypic and genetic heterogeneities of TS, which make the diagnosis and especially the management increasingly difficult. We present in this work a genetic study of TS in the Tunisian population to establish a genotype–phenotype correlation, which would be of great help for the diagnosis and the care of patients.

Patients and methods
A total of 26 unrelated Tunisian girls were included in this study. All patients underwent a complete clinical and biochemical examination as well as karyotyping. The screening for the *SRY* gene was carried out by fluorescence in-situ hybridization or by PCR.

Results
Cytogenetic results showed a prevalence of the 45,X karyotype in 46% of patients and various proportions of the other karyotypes. However, genotype–phenotype correlation revealed several discrepancies regarding the major signs and the age at diagnosis. The comparison of the approaches used for the screening of the *SRY* gene showed that karyotyping is unable to detect low 45,X/46,XY mosaicism and that it is the PCR that would be able to do, eliciting its role to make a reliable diagnosis.

Conclusion
The karyotype alone is not sufficient to make a TS diagnosis in cases of weak mosaicism, and the great heterogeneity that reigns the syndrome elicits an epigenetic and transcriptomic exploration of several genes that recently seem to be involved in the disease.

Keywords:
epigenetic, genotype–phenotype correlation, karyotype, Turner syndrome

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Introduction

Turner syndrome (TS), reported for the first time in 1938 by internist Henry Turner, is the most common sex chromosome abnormality in women and occurs in approximately one in 2500 live births [1]. It is often characterized by the absence of all or part of a normal second sex chromosome, leading to three consistent phenotypic features always present in patients with TS including short stature, ovarian dysgenesis, and dysmorphic features with webbed neck, nail dysplasia, high-arched palate, and short fourth metacarpal [2,3]. Thus, the majority of patients are treated with growth hormone and estrogen replacement therapies [4]. Moreover, several phenotypic features are associated with the major signs, giving rise to the striking clinical heterogeneity of the TS. These features include cardiac abnormalities, thyroid and autoimmune diseases, urological and bone malformations, impaired glucose tolerance, and hearing loss [5–7].

Karyotyping is the first screening reflex of TS. It could be prenatal via amniocentesis and chronic villous

sampling based on intrauterine growth retardation and fetal edema on ultrasonography, or on abnormal results of fetal karyotyping performed because of advanced maternal age. In postnatal period, karyotype is performed from peripheral blood leukocytes. A more sensitive molecular diagnosis can also be carried out by fluorescence in-situ hybridization (FISH) and PCR techniques [7].

TS is defined by a partially or completely absent X-chromosome. The majority of females with TS have a non-mosaic 45,X karyotype [8,9]. Several mosaic karyotypes exist with a cell population mix such as 46,XX and 45,X, short or long arm deletion, ring X, and isochromosome of the long arm [10]. In rare cases of mosaicism not exceeding 7%, genetic material from the Y-chromosome: the *SRY* gene (sex regulation on the Y) or even the entire Y-chromosome may be present in some cells. Moreover, a rare but very informative class of TS includes patients who have deletions of the Y-chromosome, which removes the *SRY* gene; these individuals develop as women. Based on this finding, it was hypothesized that copies of TS genes are also present on the Y-chromosome [11].

The present work focuses on the cytogenetic and molecular results of a retrospective study conducted on 26 unrelated Tunisian female with TS. The purpose of this study was to establish clinical and genetic diagnoses and to look for a genotype–phenotype correlation, allowing adequate treatment, care, and counseling.

Patients and methods

Patients

We retrospectively enrolled 26 unrelated Tunisian patients who presented to the Endocrinology Department at the Hédi Chaker University Hospital for TS suspicion. Patients less than or equal to 18 years old were considered girls, whereas the others were considered women. The cohort shows a great phenotypic variability, making the diagnosis quite complicated. All patients underwent thorough clinical, cytogenetic, and molecular explorations.

Ethical approval

The present study was approved by the local Ethics Committee of CHU Hédi Chaker of Sfax, Tunisia under the approval number 81921/22 and was conducted with the Code of Ethics of the World Medical Association, according to the principles expressed in the Declaration of Helsinki. Required samples and data were collected from patients and controls after informed consent and were anonymized.

Methods

Clinical diagnosis

The physicians recorded findings on based on detailed questions relating to birth history and presenting characteristics, including cardiac, renal, endocrine systems, ear nose and throat, skin and skeletal system, as well as psychosocial and metabolic problems that were relevant to TS. Cardiac and renal findings were based on ultrasound examinations. Thyroid diseases were evaluated by testing for anti-thyroid peroxidase antibody, anti-thyroglobulin antibody, thyroid function tests, and thyroid sonography.

Karyotyping

Karyotyping was performed on the 26 patients according to standard methods [12,13]. Peripheral blood samples were collected from each patient in sodium heparin tubes. Lymphocytes were cultured for 72 h in RPMI-1640 with phytohemaglutinin stimulation at 37°C, and colchicine was added before harvest to obtain prometaphase chromosomes. The cultured lymphocytes were treated with hypotonic solution (0.075 M potassium chloride) and then fixed in Carnoy's fixative (methanol : acetic acid 03 : 1 v/v). After staining with the Giemsa, chromosomal analysis was performed with GTG banding on at least 30 metaphases for each patients and compared with control. The chromosomal abnormalities were reported in accordance with the current standard international nomenclature [14].

Fluorescent in-situ hybridization

FISH approach involves the hybridization of fluorescent specific probes with DNA from patients, allowing a high specificity compared with karyotyping. In our study, FISH was performed for the screening of the *SRY* gene in 20 patients for whom DNA was not available, using four probes: two Y-chromosome specific probes complementary to the *SRY* gene (SRY14 and RPS4Y1) and two technique internal control probes (*DXZ1* and *DYZ1*). The DXZ1 probe is specific for X-chromosome, whereas the DYZ1 probe is specific for the Y-chromosome heterochromatin [15].

DNA extraction

Genomic DNA extraction was done for six patients using a standard protocol [16]. DNA isolation was performed using the TSNT lysis buffer (1% Triton, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA) followed by phenol–chloroform extraction. DNA was then diluted and stored at 4°C before analysis.

PCR

Duplex PCR was carried out in TS3, TS6, TS7, TS8, TS13, and TS14 using two pairs of primers to amplify simultaneously both *SRY* and *ZFX* genes regions. The primers used for the *SRY* gene are 5' GAATATT CCCGCTCTCCGGA 3' and 5' GCTGGTGCTCC ATTCTTGAG 3' whereas those used for the *ZFX* gene are 5' ACCRCTGTACTGACTGTGATT ACAC 3' and 5' GCACYTCTTTGGTATCYG AGAAAGT 3', leading to amplified fragments of 472 and 495 bp, respectively. Expected PCR products were electrophoresis on a 5% polyacrylamide gel in tris-acetate EDTA buffer [16].

Statistical analysis

All data were collected, revised, and processed using Microsoft Excel 2007. All data were presented as numbers and percentage.

Results

Clinical findings

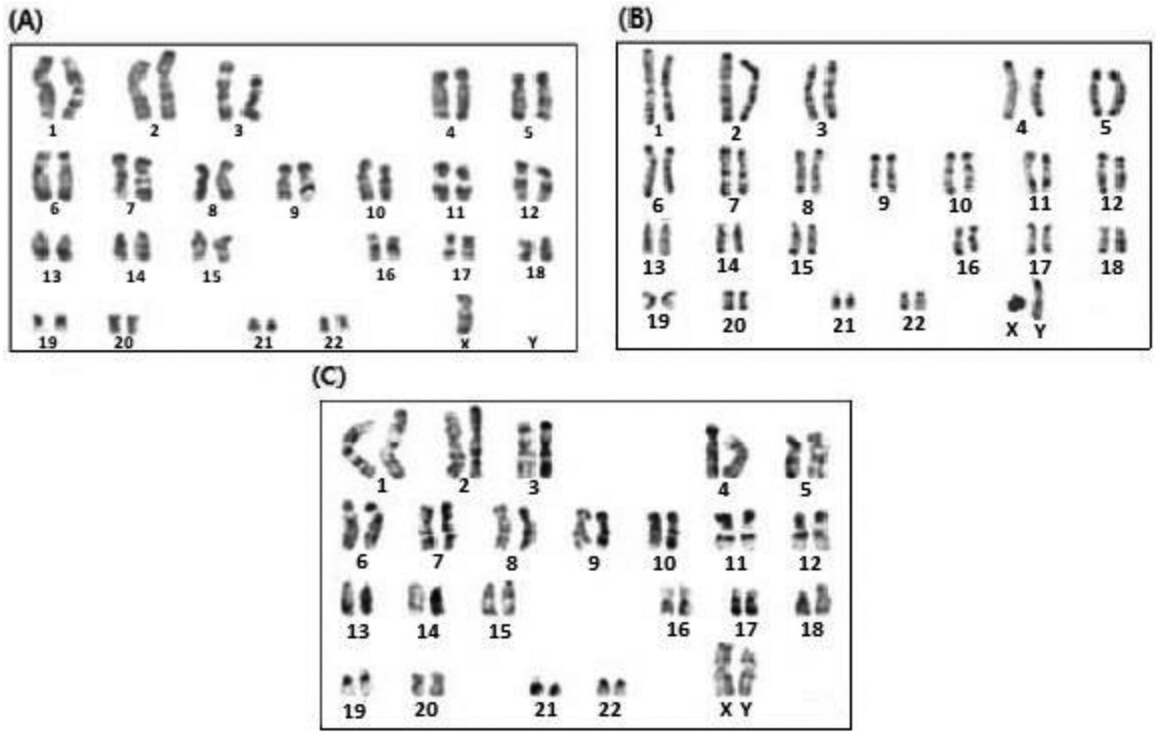
The exhaustive clinical explorations of the studied cohort showed a striking phenotypic heterogeneity, as summarized in Table 1.

Table 1 Clinical features and karyotypes for the studied patients

Patient ID	Age at presentation	Clinical features	Karyotype
TS1	15 years	GR, POF, DF, gonadal dysgenesis, and adrenal tumor	(45, X)
TS2	15 years	GR, POF, DF, delayed puberty, and anterior pituitary insufficiency.	(45, X)
TS3	17 years	GR, POF, DF, delayed puberty, and facial paralysis	(46,X,i(Xq))mosaic
TS4	12 years	GR, DF, hyperthyroidism, and obesity	(45, X)
TS5	18 years	GR, POF, DF, and primary amenorrhea	[46,X,i(Xq)] mosaic
TS6	5 years	GR, POF, gonadal dysgenesis, and spastic paraplegia	(45,X/46,XX) mosaic
TS7	44 years	GR, POF, primary amenorrhea, osteopenia, nephrolithiasis, hepatic cytolysis, and hepatic cholestasis	[46,X,i(Xq)] mosaic
TS8	19 years	GR, POF, delayed puberty, and secondary amenorrhea	45,X/46,X r(X) mosaic
TS9	44 years	DF, osteoporosis, Behcet's disease, and breast cysts	(45,X/46,XX) mosaic
TS10	16 years	GR, POF, DF, delayed puberty, hypothyroidism, goiter, Hashimoto's thyroiditis, and hyperprolactinemia	[46,X,i(Xq)] mosaic
TS11	16 years	GR, POF, DF, primary amenorrhea psoriasis, goiter, and anterior pituitary insufficiency	(45,X/46,XX) mosaic
TS12	15 years	GR, POF, DF, primary amenorrhea, and hyperprolactinemia	(45,X/46,XX) mosaic
TS13	16 years	GR, POF, DF, delayed puberty, primary amenorrhea, gonadic dysgenesis, arterial hypertension, heart disease, and aortic contraction	(45, X/46, XY) mosaic
TS14	15 years	GR, POF, delayed puberty, gonadic dysgenesis, and congenital heart disease	(45, X/46, XY) mosaic
TS15	18 years	GR, POF, DF, primary amenorrhea, gonadic dysgenesis, hyperprolactinemia, and mental retardation	(45, X)
TS16	24 years	GR, POF, DF, delayed puberty, primary amenorrhea, and hyperthyroidism	(45, X)
TS17	27 years	GR, POF, DF, delayed puberty, primary amenorrhea, osteoporosis, and single kidney	(45, X)
TS18	36 years	GR, POF, DF, delayed puberty, primary amenorrhea, and osteoporosis gonadic dysgenesis goiter	(45, X)
TS19	14 years	GR, POF, DF, delayed puberty, and primary amenorrhea	(45, X)
TS20	16 years	GR, POF, DF, delayed puberty, and primary amenorrhea	(45, X)
TS21	15 years	GR and delayed puberty	(45, X)
TS22	14 years	GR, POF, DF, delayed puberty, primary amenorrhea, hypothyroidism, hyperprolactinemia, iron deficiency, and anemia	(45,X)
TS23	14 years	GR, POF, DF, delayed puberty, primary amenorrhea, obesity, and unique kidney	(45, X)
TS24	42 years	Early menopause, primary sterility, obesity, and type 2 diabetes	(45,X/46,XX) mosaic
TS25	17 years	Delayed puberty, primary amenorrhea, and gonadal dysgenesis	(45,X/46,XX) mosaic
TS26	38 years	Early menopause, primary infertility, secondary amenorrhea, primary ovarian failure, hyperthyroidism, goiter, exophthalmos, and Graves' disease	(45,X/46,XX) mosaic

DF, dysgenesis failure; GR, growth retardation; POF, polycystic ovary failure.

Figure 1

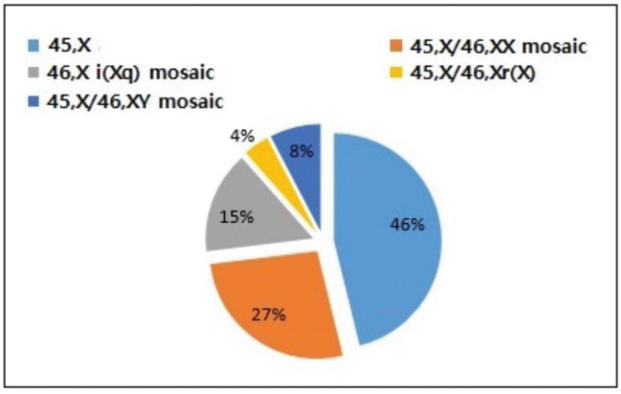


Examples of karyotypes in patients with TS. (a) 45,X karyotype in patient TS1, (b) 45,X/46,X r(X) karyotype in patient TS8, and (c) 46,X isoXq karyotype in patient TS5.

Cytogenetic results

Karyotypes of patients were classified according to numerical, structural, and both numerical and structural anomalies by a geneticist. Results showed five variant karyotypes with different frequencies (Figs 1 and 2): the non-mosaic (45,X), which is a chromosome number anomaly found in 46% of cases (n=12) (Fig. 1a, Fig. 2), whereas 27% (n=7) of patients had a mosaic karyotype, with (46,XX) and (45,X) cell lines (Fig. 1b, Fig. 2). Mosaic karyotypes with structure anomaly were also found in 4 and 15% of patients who have an X ring [45,X/46,X r (X)] and an isochromosme X [46,X,i(Xq)] karyotypes, respectively (Fig. 1c, Fig. 2). Two (8%) patients showed a mosaic (45,X/46,XY) karyotype with the presence of the Y-chromosome (Fig. 2).

Figure 2

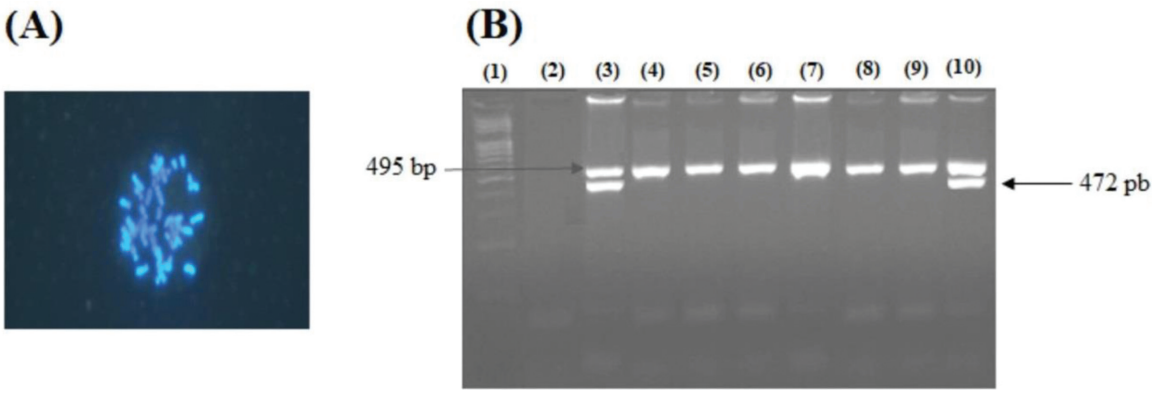


Percentages of different karyotypes found in the studied patients. We note that the (45,X) was found in 46% of cases, 27% had a mosaic 45,X/46,XX karyotype, 15 and 8% had a X [46,X,i(Xq)] and (45,X/46,XY), respectively, and only 4% had [45,X/46,X r (X)] karyotype.

Screening of the *SRY* gene: fluorescence in-situ hybridization and PCR results

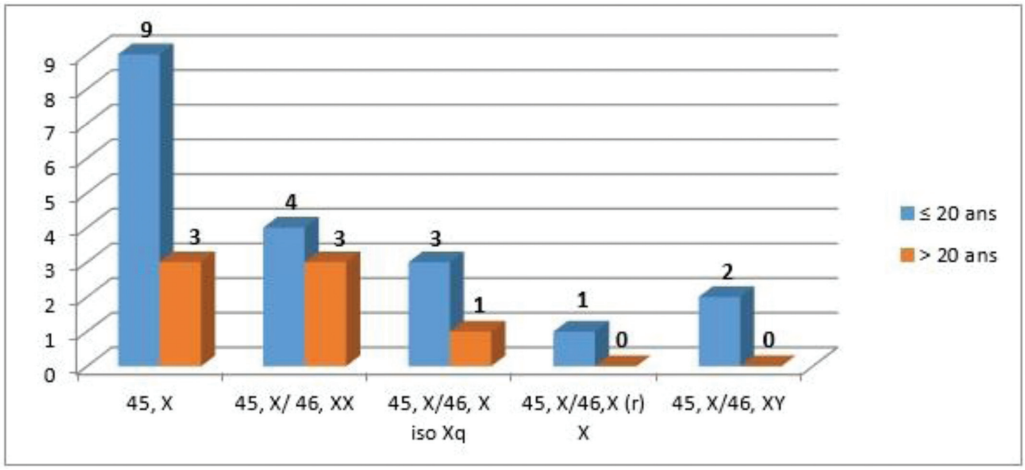
Conventional karyotyping is limited to the detection of rearrangements involving more than 5 Mb of DNA. Thus, the FISH or the PCR assays were used to search for the *SRY* gene. The FISH is a molecular cytogenetic method that can detect sequences of 100 kb–1 Mb, whereas PCR is a very sensitive and specific molecular genetic strategy that can detect and amplify low quantity or even traces of DNA. In our cohort, the FISH was performed in 20 patients. None of the patients showed the presence of the *SRY* gene following the absence of any fluorescent signal with the complementary probes used (Fig. 3a). Besides, PCR results performed in patients TS3, TS6, TS7,

Figure 3



Molecular screening of the *SRY* gene by FISH and PCR. (a) Absence of the *SRY* gene in TS2 by FISH. (b) Gel electrophoresis of co-amplified PCR products of *SRY* (472 bp) and *ZFX* (495 bp) genes. Results showed the absence of the *SRY* gene in TS6, TS7, TS8, TS13, and TS14 (lane 4, 5, 6, 7, 8, and 9, respectively) and its presence in a control man (lane 3) and in TS3 (lane 10). The size marker is a 100-bp DNA ladder from Fermentas (lane 1) and lane 2 is a negative internal control. FISH, fluorescence in-situ hybridization.

Figure 4



Histogram illustrating the distribution of patients according to the karyotype and the age at presentation. Results showed that the age of diagnosis is less than or equal to 20 years for the (45,X), [45,X/ 46,X r (X)], [X (46,X,i(Xq)], and the (45,X/46,XY) karyotypes, whereas for the seven patients with a (45,X/46,XX) mosaic karyotype, four of them were consulted before the age of 20 years and three were consulted after their 20 s.

TS8, TS13, and TS14 showed that only TS3 was positive for the *SRY* gene (Fig. 3b).

Correlation between the karyotype and the age of diagnosis

We first tried to classify the patients according to two criteria: the karyotype and the age of diagnosis. The results showed that the age of diagnosis was clearly less than or equal to 20 years for the [45,X), (45,X/46,X r (X)], [X (46,X,i(Xq)], and (45,X/46,XY) karyotypes. However, for the seven patients with a (45,X/46,XX) mosaic karyotype, we observed that four of them consulted before the age of 20 years during their first decade, whereas three consulted after their 20 s (Fig. 4). By re-examining the karyotypes, we noticed that these four patients had a mosaicism with a (45,X) cell line majority, whereas the three patients who consulted after their 20 s had a low mosaicism with a (46,XX) majority population.

Karyotype–phenotype correlation

A karyotype–phenotype correlation was establish in our cohort based on the results summarized in Table 2, leading to these findings:

We first noted that dysmorphic signs, primary ovarian failure, and delayed growth are present in patients, with respective percentages of 85, 81, and 62%. Besides, we observed that the delayed puberty and the primary amenorrhea are present in patients, with percentages of 61 and 54%, respectively. Thyroid diseases (hypothyroidism and hyperthyroidism) are also

Table 2 Distribution of the major and associated signs of Turner syndrome in our cohort according to the patient's karyotypes

	45,X (12)	45,X/ 46, XX (7)	45,X/46, X ring(X) (1)	45,X/46, X iso Xq (4)	45,X/46, XY (2)	Total (26) [*n* (%)]
Growth retardation	12	3	1	4	2	22 (85)
Dysmophicfeatures	11	4	1	4	1	21 (81)
Primaryovarianfailure	8	2	1	3	2	16 (62)
Delayed puberty	7	4	1	2	2	16 (62)
Primary amenorrhea	7	3	0	3	1	14 (54)
Secondary amenorrhea	1	1	1	0	0	3 (12)
Spaniomenorrhea	1	1	0	0	0	2 (7.7)
Earlymenopause	0	2	0	0	0	2 (7.7)
GH deficiency	1	2	0	1	0	4 (15.4)
Hypothyroidism/hyperthyroidism	3	2	0	2	0	7 (27)
Autoimmune diseases	0	1	0	1	0	2 (7.7)
Osteoporosis	2	1	0	1	0	4 (15.4)
Gonadal dysgenesis	4	3	0	0	2	9 (34.6)
Heartdiseases	0	0	0	0	2	2 (7.7)
Obesity	2	1	0	0	0	3 (11.5)
Single kidney	2	0	0	0	0	2 (7.7)

frequent in 25% of patients and are in most cases of autoimmune origin; the Graves' disease is associated with hyperthyroidism, whereas the Hashimoto's thyroiditis is associated with hypothyroidism. The other associated signs are present at low percentages, ranging from 0% for the digestive abnormalities to 30% for the gonadal dysgenesis. The growth retardation was absent in four patients with 45,X/46,XX karyotype, and patients with 45,X/46,XY mosaic karyotype showed the presence of gonadal dysgenesis. Moreover, we found that the homogeneous 45,X is the only karyotype in our cohort that is associated with the incidence of having a single kidney in two patients presenting the most severe phenotype.

Discussion

The principal challenge in TS consists in developing a relationship between the chromosomal abnormalities and the clinical findings, which would be of great interest for the diagnosis and especially for the management of patients. Studies that have examined this correlation have been disappointing; this was confirmed in our study, which showed some concordances as well as some discrepancies with the data of the literature. In fact, we found that the frequency of karyotypes is 46% for the (45,X), 27% for the (45,X/46,XX), 15% for the Xq isochromosome, and 22% for the other formula, thus corresponding with the data from the literature. Indeed, in a large Danish series, 45% of patients had a 45,X karyotype, 11% had Xq isochromosome, and 44% had other karyotypes [17]. The high frequency of the 45,X karyotype was also described in American and Belgian cohorts, with frequencies of 54 and 47%, respectively [2,18].

Moreover, all the previous studies considered dysmorphic signs, delayed growth, and primary ovarian failure found in our patients, with respective frequencies of 85, 81, and 62% as the three major signs of TS. Yet, the prevalence of the delayed puberty and the primary amenorrhea, with percentages of 61 and 54%, respectively, suggests that these signs should be considered and included for the diagnosis of TS even in the absence of one of the major signs. Besides, a difference in the age of consultation was observed among patients with 45,X/46,XX mosaic karyotype with an early diagnosis for those with a (45,X) cell line majority, eliciting a correlation between the age of diagnosis and the rate of abnormal cell population.

Furthermore, it has been long accepted that growth delay is the only clinical finding invariably associated with the 45,X karyotype, and it is the only phenotypic feature present in virtually 100% of TS patients [11]. The absence of growth retardation in four patients with 45,X/46,XX mosaic karyotype elicits that the absence of short stature should not exclude the TS diagnosis.

Moreover, homogeneous 45,X is the only karyotype in our series that is associated with the incident of having a single kidney in two patients presenting the most severe phenotype. Thus, the finding confirms another study, which found that structural malformations of the kidney occur more frequently in patients who have a 45, X karyotype, whereas malformations of the collecting

system occur more frequently in those with mosaic 45, X/46 karyotypes [19].

Although specifically male, the Y-chromosome can be found in female patients and more particularly in women with TS. It may be a Y-chromosome that has a mutated *SRY* gene or that has lost it by deletion, resulting usually in isolated gonadal dysgenesis. To confirm one of the two hypothesis, FISH or PCR assay is needed. The results revealed that both TS13 and TS14 patients who have mosaic (45,X/46,XY) karyotype do not show the presence of the *SRY* gene by PCR. This finding approves the presence of the Y-chromosome with a deleted *SRY* gene in these patients, which often leads to chromosomal structural anomaly such as carrying two centromeres, which makes them unstable during mitosis. Conversely, one patient who did not show a Y-chromosome on the karyotype had positive PCR for the *SRY* gene. This clearly confirms the great specificity of the molecular approach and the inadequacy of the cytogenetics to establish a reliable diagnosis of TS [20,21].

The absence of a karyotype–phenotype correlation raises several questions as to the origin of the great phenotypic heterogeneity that reigns the TS. We can suggest in this context the involvement of genetic and epigenetic factors that can modulate the TS phenotype. Indeed, in a typically developing female with a normal complement of 46 chromosomes, one of the X chromosomes is inactivated during early embryonic development, a phenomenon known as dosage compensation or lyonization [22,23]. This epigenetic mechanism functions to equalize the dosage of X-linked genes between females and males. However, 15% of genes on the 'inactive' X-chromosome in females escape inactivation [24,25]. TS is likely to be a syndrome of the haploinsufficiency of developmental genes, resulting in partial or complete absence of these genes that escape inactivation [26]. Among them, the homeobox *SHOX* gene located on the pseudoautosomal region of the X-chromosome seems to be associated with skeletal abnormalities and short stature in TS [27]. Genetic studies have even shown that growth retardation in patients with TS is not due to the growth hormone deficiency but largely to the haploinsufficiency of the *SHOX* gene [28,29]. Such mechanism was also recently reported for the *TIMP1* gene encoding for a metalloproteinase inhibitor which participates in the development of the aortic valve and its tissue integrity. The hemizygosity of *TIMP1* on the X-chromosome and variants of its autosomal paralogue *TIMP3* on chromosome 22 significantly increase the risk of aortopathy in patients with TS [30]. Other candidate genes are currently under investigation. Methylation is the second epigenetic aspect influencing the TS condition. It is involved in genomic imprinting to determine which X-chromosome and genes will be inactivated. Imprinting depends on DNA and histone methylation on one of the two parental X-chromosome [31]. Although the issue remains controversial, a limited number of studies investigating imprinting effects in TS have demonstrated a possible influence on cognitive-behavioral phenotype. In fact, individuals who retain the maternal X-chromosome (Xm) show greater impairments with altered neurodevelopment in temporal and occipital regions compared with those with the paternal X-chromosome (Xp) [32,33]. Moreover, several other candidate genes seem to be overexpressed by hypomethylation in patients with TS compared with normal patients [34]. Among these genes, some authors found that the *KDM5C* and the *IL3RA* genes are involved in neurological and autoimmune impairment [35,36], respectively; the *CSF2RA* gene is responsible for early intrauterine lethality [37]; and the *TIMP1* and the *KDM6A* genes are related to cardiac impairment [34,38].

Conclusion

The present study can conclude that TS is a complex pathology ruled by a large phenotypic heterogeneity emerging from a genetic and epigenetic imprint. Future investigations of TS should include continued genetic studies such as microarray analyses and determination of candidate genes for both physical and cognitive features. Multimodal, interdisciplinary studies will be essential for identifying optimal, syndrome-specific interventions for improving the lives of individuals with TS.

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Conflicts of interest

There are no conflicts of interest.

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