ORIGINAL ARTICLES

Detection of Turner Syndrome by Quantitative PCR of *SHOX* and *VAMP7* Genes

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Turner Syndrome (TS) is an unfavorable genetic condition with a prevalence of 1:2500 in newborn girls. Prompt and effective diagnosis is very important to appropriately monitor the comorbidities. The aim of the present study was to propose a feasible and practical molecular diagnostic tool for newborn screening by quantifying the gene dosage of the *SHOX*, *VAMP7*, *XIST*, *UBA1*, and *SRY* genes by quantitative polymerase chain reaction (qPCR) in individuals with a diagnosis of complete X monosomy, as well as those with TS variants, and then compare the results to controls without chromosomal abnormalities. According to our results, the most useful markers for these chromosomal variants were the genes found in the pseudoautosomic regions 1 and 2 (PAR1 and PAR2), because differences in gene dosage (relative quantification) between groups were more evident in *SHOX and VAMP7* gene expression. Therefore, we conclude that these markers are useful for early detection in aneuploidies involving sex chromosomes.

Introduction

TURNER SYNDROME (TS) AFFECTS 1 in 2500/3000 live-born girls and is characterized by short stature, gonadal dysgenesis, *pterygium colli*, *cubitus valgus*, and a low hairline (Wiedemann and Glatzl, 1991; Ranke and Saenger, 2001; Pinsker, 2012). It is caused by partial or total loss of the second sex chromosome (Ford *et al.*, 1959; Sybert and McCauley, 2004). Complete monosomy X (45,X) represents 50–60% of all cases, while mosaics of two or more cell lines and structural aberrations (mostly Xq isochromosomes) account for the remaining 40–50% (Sybert and McCauley, 2004).

The X chromosome has 155 megabase pairs (Mb) and contains ~1000 genes (Bianchi *et al.*, 2012), while the Y chromosome (60 Mb) contains only 104 protein-coding sequences out of a total of over 200 genes (Li *et al.*, 2008). Inactivation of the majority of one X chromosome in females leads to a functional 1n dosage of X-linked genes in both genders. The pseudoautosomal regions (PAR)1 and 2 homologous sequences, which are present on both the X and Y chromosomes, escape from X chromosome inactivation. Therefore, the 24 genes present in the 2.6 Mb PAR1 (Mangs and Morris, 2007) and the 4 genes in the 320 kb PAR2 have a functional gene dosage of 2n in both genders. Hence, the etiology of TS lies in the haploinsufficiency of genes located in the PAR1 (Zhong and Layman, 2012).

Guidelines of the American College of Endocrinology for the management of patients with TS emphasize the benefit of early detection through newborn screening methods (Bondy *et al.*, 2007). However, only 20–30% of TS patients are diagnosed during the first year of life and another 25% are diagnosed in adulthood (Massa *et al.*, 2005; Stochholm *et al.*, 2006). In Mexico, less than 15% are diagnosed during the first year of life. Early detection allows identification of malformations of the cardiovascular system and prevention or management of short stature, hearing problems, and learning difficulties (Bondy *et al.*, 2007; Pinsker, 2012). Moreover, it is feasible to diminish the risk of infertility in some individuals by cryopreserving oocytes while follicles are still viable (Kavoussi *et al.*, 2008; Borgstrom *et al.*, 2009).

The dosage reduction of genes located in PARs1 and 2, which is inherent to the complete or partial loss of the second sex chromosome, can be sensitively quantified by quantitative polymerase chain reaction (qPCR), as reported for the *ARSE* gene in Xp22.33 (Rocha *et al.*, 2010).

The aim of the present study was to determine the dosage of two genes located in the pseudoautosomal regions (short-stature homeobox [*SHOX*] and vesicle-associated membrane protein 7 [*VAMP7*]), two X-specific genes (ubiquitin-like modifier activating enzyme 1 [*UBA1*] in p, and X inactive-specific transcript [*XIST*] in q), the sex determining region Y (*SRY*) gene by qPCR in TS patients, and to compare the results to those for the gene dosage of men and women with a

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normal karyotype. These findings were used to evaluate the effectiveness of these measurements as an affordable method for TS screening.

Materials and Methods

Population

We performed a descriptive, comparative, nonblinded study that included 27 TS patients who were karyotyped between 2005 and 2012 by the Genetics Department, University Hospital, ''Dr. Jose E González'' of the Universidad Autónoma of Nuevo León in Monterrey, México. Volunteer subjects with a normal karyotype (10 females and 10 males)

were recruited from the same institution and used as controls. Written informed consent was obtained from all subjects or legal guardians, and the Health Research Ethics Board of the UANL Medical School approved the study (Approval No.: GN-11-004).

The subjects were divided into four groups according to their karyotype: (a) 45,X (X monosomy); (b) 46,XY (male); (c) 46,XX (female); and (d) TS variants (mosaicism and structural aberrations).

Sample collection

Biological blood samples. Four milliliters of peripheral venous blood was collected in a heparin-treated tube from

TABLE 1. RELATIVE QUANTIFICATION RESULTS FOR THE *SHOX*, *XIST*, *VAMP7*, *SRY*, *UBA1* GENES

Case	Karyotype	RQ SHOX	RQ XIST	RQ VAMP7	RQ SRY	RQ UBA1
1	45,X	0.531	0.474	0.537	0	0.559
2	45,X	0.571	0.501	0.491	0	0.502
3	45,X	0.532	0.561	0.609	0	0.462
4	45,X	0.572	0.38	0.47	0	0.649
5	45,X	0.505	0.462	0.451	0	0.521
6	45,X	0.556	0.513	0.609	0	0.564
7	45,X	0.494	0.549	0.531	0	0.519
8	45,X	0.545	0.512	0.534	0	0.508
9	45,X	0.479	0.51	0.525	0	0.513
10	45,X	0.624	0.638	0.637	0	0.46
11	45,X	0.472	0.516	0.645	0	0.53
12	45,X	0.518	0.421	0.492	0	0.722
13	45,X,inv (7)(q31.2-pter)	0.509	0.515	0.479	0	0.537
14	45,X[6]/46,XX[24]	1.169	0.733	0.479	0	0.824
15	45,X[2]/46,XX[28]	0.8	0.871	0.777	0	1.602
16	45,X[18]/46,XX[12]	0.623	0.605	0.629	0	0.611
17	45,X[12]/46,XX[18]	0.535	0.718	0.761	0	0.533
18	45,X[13]/46,XX[7]	0.59	0.689	0.711	0	0.702
19	45,X[6]/47,XXX[24]	1.016	1.108	1.084	0	1.079
20	45,X[10]/49,XXXX[3]/46,XX[86]	0.99	0.906	0.899	0	1.034
21	45,X[6]/47,XXX[24]	0.703	0.63	0.616	0	0.762
22	45,X[20]/46,XY[10]	0.809	0.509	0.792	0.561	0.542
23	45,X[17]/46,XY[3]	0.859	0.484	0.458	1.784	0.549
24	45,X[17]/46,X,r[13]	0.506	0.688	0.473	0	0.694
25	45,X[5]/46,X,i(X)(q10)[25]	0.53	1.053	1.146	0	0.514
26	45,X[6]/46,X,i(X)(q10)[24]	0.527	1.012	1.097	0	0.558
27	46,X,i(X)(q10)	0.5	1.026	1.535	0	0.973
28	46,XX	1	1.062	1.083	0	1.012
29	46,XX	1	1	1	0	1
30	46,XX	1	1	1	0	1
31	46,XX	1	1	1	0	1
32	46,XX	1	1	1	0	1
33	46,XX	0.908	1.111	1.039	0	0.999
34	46,XX	1	1	1	0	1
35	46,XX	1	1	1	0	1
36	46,XX	1	1	1	0	1
37	46,XX	1	1	1	0	1
38	46,XY	1.069	0.513	1.127	0	0.536
39	46,XY	0.952	0.56	1.105	2.737	0.533
40	46,XY	1.025	0.561	1.103	0	0.548
41	46,XY	1.052	0.558	1.613	0	0.495
42	46,XY	0.994	0.491	0.971	0	0.507
43	46,XY	0.959	0.506	1.097	3.897	0.511
44	46,XY	1.005	0.487	1.104	0	0.524
45	46,XY	0.981	0.472	1.016	0	0.552
46	46,XY	1.053	0.488	0.999	0	0.547
47	46,XY	1.169	0.512	1.164	1.007	1.042

RQ, relative quantification.

each participant for G-banding karyotype (Barch *et al.*, 1997) in our laboratory, which is accredited by the College of American Pathologists (CAP). Only four patients and the control subjects were karyotyped during this study. Twenty-three patients who had been previously karyotyped and diagnosed with TS were invited to participate, and a 4-mL blood sample was collected in EDTA tubes to obtain total genomic DNA. Extraction was conducted using an automated QIAcube® instrument and QIAamp® DNA Mini and Blood Mini Extraction Kits (Qiagen, Valencia, CA).

Gene selection. Five probe-on-demand assays (Applied Biosystems, Foster City, CA) were used to measure the dosage of five genes: *SHOX* at PAR1, *VAMP7* at PAR2, *UBA1* at Xp11.23, *XIST* at Xq13.2, and *SRY* at Yp11.3 (Table 1). The *RNase P* gene was used as an autosomal reference control.

Real-time PCR analysis. The dosage of each gene was measured with the StepOnePlus™ System (Applied Biosystems) and TaqMan Genotyping Master Mix 2X (Applied Biosystems) following the manufacturer's instructions. Relative quantification (RQ) values were obtained using the Delta CT comparison method ($\Delta\Delta C_T$) (Livak and Schmittgen, 2001). Amplification reactions were performed in triplicate with determined reproducibility. In TS patients, an RQ <1 for *SHOX* and *VAMP7* was expected.

Statistical analysis

Descriptive statistics were applied to the RQ values of each gene. ANOVA was used to analyze intergroup differences between gene dosages, which were determined by the mean RQ values of each gene. Differences were considered statistically significant when the *p*-value was <0.05.

Results

Of the 47 samples, 20 were from the control group (10 46,XY and 10 46,XX) and 27 from TS patients. Among the latter, 13 (48%) had monosomy X and 14 had a TS variant, with 45,X/46,XX mosaicism being the most frequent (19%) karyotype (Table 1).

The RQ value of the *SHOX* gene in patients with TS ranged from 0.50 (minimum) to 0.85 (maximum) and it ranged from 0.97 to 1.07 in the control group (Table 1). The *VAMP7* gene presented an RQ range from 0.47 to 0.57 in patients diagnosed with complete X monosomy (Table 1) and from 0.57 to 0.99 for the TS chromosomal variants. The RQ values from the control group ranged from 0.99 to 1.25. The RQ values for the *XIST* gene in the patients with complete X monosomy ranged from 0.46 to 0.54 and from 0.66 to 0.90 in the TS variant group. In contrast to the patients, the RQ for the control group ranged from 0.98 to 1.04 in female subjects with a 46,XX karyotype and from 0.49 to 0.53 in males with a 46,XY karyotype (Table 1). In the case of the *UBA1* gene, the RQ in patients with complete X monosomy was from 0.49 to 0.58,
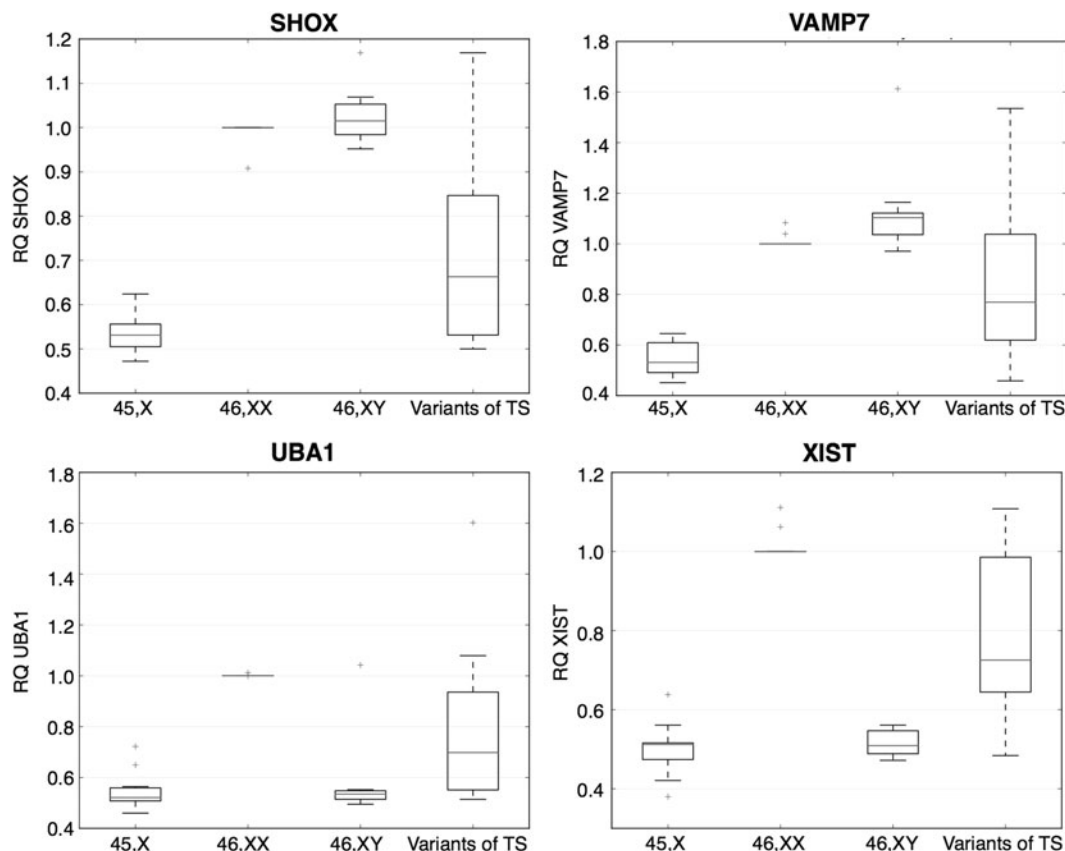

FIG. 1. Box and whisker plot of relative quantification (RQ) results from the four groups evaluated.

and in the TS variant group, the RQ values ranged from 0.60 to 0.95. The control group RQ range for the same gene was from 0.99 to 1.00 for 46,XX and from 0.46 to 0.69 for 46,XY. The *SRY* gene RQ in patients with complete X monosomy was 0.0, ranging from 0.47 to 0.66 in the TS variant group. For the controls, the RQ for the *SRY* gene was 0.0 in the 46,XX female subjects and from 0.97 to 1.09 in male 46,XY subjects.

The ANOVA showed significant differences ($p < 0.05$) in the gene dosage for *SHOX*, *XIST*, *UBA1*, and *VAMP7* between the study groups and the controls. The most useful markers for the TS variant group (mosaicism and structural aberration) were those found in PAR1 and PAR2 and *SHOX* and *VAMP7*, which showed more consistent results and a greater ability to discern between the TS patients versus controls (Fig. 1).

In the descriptive analysis, the RQ ranges of *SHOX* and *VAMP7* obtained from the TS groups showed a difference of more than ± 3 standard deviation from that of the control group. The RQ cutoff was established at 0.8, with a sensitivity and a specificity of 100% in patients with complete monosomy X and a sensitivity of 64% and a specificity of 100% for the TS variants. If we analyzed mosaic TS (45,X/46,XX) only, the sensitivity lowers to 83% and to 64% in TS variants.

All of the gene markers in patients with different chromosomal variants were evaluated to analyze whether the chromosomal formula could be identified without a conventional karyotype. Using individuals with a 46,XX karyotype as a reference, a difference between the gene dose of *XIST* and *UBA1* was found; however, when compared to 46,XY individuals, who are hemizygous for these genes, the RQ was similar to that found in most patients with TS. Thus, the RQ for *XIST* and *UBA1* was not considered to be useful for analysis and diagnosis (Fig. 1). The *SRY* gene is only of qualitative utility because it identifies individuals with a 46,XY chromosomal formula.

Discussion

Early diagnosis of TS leads to proper treatment of complications and can aid in avoiding or reducing deleterious consequences in adult life. The frequent delay in the diagnosis of TS justifies the search for methodologies that allow prompt diagnosis of partial or complete, homogeneous or mosaic, and X chromosome monosomy. In the present study, we analyzed 27 patients with TS to determine the gene dosage of *SHOX*, *VAMP7*, *XIST*, *UBA1*, *SRY*, and *RNase P* in 47 individuals. The group with complete monosomy X represented 48% of all samples in the study group. Of the remaining TS patients, 19% were mosaic with 45,X/46,XX, followed by other mosaics and structural aberrations present in similar proportions to those reported in the literature (Sybert and McCauley, 2004).

In our study, the differences between RQ means in the patients with complete monosomy and control subjects were significantly different. Similar findings have been reported by Rocha *et al.* (2010).

Overall, the patients had half of the dosage (compared to 46,XX women) in all X chromosome genes analyzed and the absence of the *SRY* gene. If the analysis of the studied genes is used as a screening test in a population, it will differentiate between individuals with a 46,XY karyotype and TS patients. Detection of the *SRY* gene is considered of great importance in patients with mosaic forms of TS (Coto *et al.*, 1995; Mendes *et al.*, 1999; Gravholt *et al.*, 2000). The analysis of

the RQ for the *SRY* gene in the present study will permit the detection of variants of TS with the presence of a Y chromosome (this occurs in 3–5% of TS cases), which increases the risk for gonadal tumors, especially gonadoblastoma (Osipova *et al.*, 1998). Therefore, we believe that including this gene (*SRY*) in our analysis provides a more useful tool for the study of patients with TS.

Patients with mosaic 45,X/46,XY had a decreased RQ for *SHOX* and *VAMP7* compared to normal males (46,XY).

Differences in the mean RQ values observed in samples with chromosomal variants were not as clear-cut, especially in the case of mosaics with a 45,X clone present below 20% (cases 14 and 20) and in mosaics with polysomy X (e.g., case 19) that presented an RQ ≥ 1. We think that the extra X chromosome compensates for the monosomy X in other cells. As expected, the most evident gene dosage alterations were observed in *SHOX* and *VAMP7*, which thereby appear to be reliable and useful markers in diagnosing TS (Fig. 1). Detection sensitivity for the different TS variants altogether was 64%, but when only mosaic forms were analyzed (45,X/46,XX), sensitivity increased up to 83%. It is important to mention that this genotype is the second most common after monosomy (Sybert and McCauley, 2004; Davenport, 2010).

Despite the low number of participants—a limitation of our study that should be addressed to increase the statistical power—the use of qPCR to determine the dosage of *SHOX* and *VAMP7* appears to be a useful, quick, and affordable neonatal screening test in infants with clinical suspicion of TS.

The delay in TS diagnosis is a problem, and methodologies that allow early diagnosis of patients, including those with chromosomal variants, are needed. Guidelines of the American College of Endocrinology for the management of patients with TS have addressed the potential benefit of designing a newborn screening method for the detection of these patients (Bondy *et al.*, 2007). We propose the quantification of the gene dose of *SHOX* and *VAMP7* as a screening tool for the diagnosis of newborns with TS.

Author Disclosure Statement

No competing financial interests exist.

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