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ARTICLE

Structural and numerical changes of chromosome X in patients with esophageal atresia

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Esophageal atresia with or without tracheoesophageal fistula (EA/TEF) is a relatively common birth defect often associated with additional congenital anomalies such as vertebral, anal, cardiovascular, renal and limb defects, the so-called VACTERL association. Yet, little is known about the causal genetic factors. Rare case reports of gastrointestinal anomalies in children with triple X syndrome prompted us to survey the incidence of structural and numerical changes of chromosome X in patients with EA/TEF. All available ($n = 269$) karyotypes of our large (321) EA/TEF patient cohort were evaluated for X-chromosome anomalies. If sufficient DNA material was available, we determined genome-wide copy number profiles with SNP array and identified subtelomeric aberrations on the difficult to profile PAR1 region using telomere-multiplex ligation-dependent probe amplification. In addition, we investigated X-chromosome inactivation (XCI) patterns and mode of inheritance of detected aberrations in selected patients. Three EA/TEF patients had an additional maternally inherited X chromosome. These three female patients had normal random XCI patterns. Two male EA/TEF patients had small inherited duplications of the XY-linked *SHOX* (Short stature HOmeoboX-containing) locus. Patients were small for gestational age at birth (<P5) and had additional, mostly VACTERL associated, anomalies. Triple X syndrome is rarely described in patients with EA/TEF and no duplications of the *SHOX* gene were reported so far in these patients. As normal patterns of XCI were seen, overexpression of X-linked genes that escape XCI, such as the *SHOX* gene, could be pathogenic by disturbing developmental pathways.
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INTRODUCTION

Esophageal atresia (EA) with or without tracheoesophageal fistula (TEF) is a relatively common birth defect affecting approximately 1:3500 newborns. These newborns can have a heterogeneous phenotype, some have EA and/or TEF as an isolated defect and others have more anomalies, predominantly VACTERL (vertebral, anal, cardiovascular, tracheoesophageal, renal and limb) associated.[1]

EA/TEF is a variable feature in several genetic syndromes, for example, Feingold (*MYCN*), CHARGE (*CHD7*), Anophthalmia-Esophageal-Genital (AEG) syndrome (*SOX2*) and Fanconi anemia.[2] In addition, the genetic defects in these syndromes and other putative causal genetic aberrations are described in EA/TEF patients. Although there are chromosomal hotspots, these aberrations are mostly scattered across the genome.[2]

Structural and numerical chromosome abnormalities affecting sex chromosomes have been described in patients with congenital malformations.[3–5] These defects are rare in patients with EA/TEF, although EA/TEF is a variable feature in patients with Opitz G syndrome (*MID1*) and VACTERL association with hydrocephalus (*FANCB*).[1,2]

There are several reports describing X-chromosome duplication in association with gastrointestinal anomalies.[6] This prompted us to evaluate retrospectively the cytogenetic results in our EA/TEF cohort.

We identified three patients with a triple X karyotype, strengthening the relationship of gastrointestinal anomalies and X-chromosome triplication. In addition to classical karyotyping, we examined the patient DNA with telomere-multiplex ligation-dependent probe amplification (MLPA) and microarrays for structural X-chromosome abnormalities. These molecular–genetic studies revealed Short stature HOmeoboX-containing gene (*SHOX*) duplications in two additional patients with EA, TEF and limb anomalies.

We hypothesize that genes on X and/or genes that escape X-chromosome inactivation (XCI) could influence essential developmental pathways in limb and foregut development.

PATIENTS AND METHODS

Patient population

This study was approved by the Medical Ethical Review Board of Erasmus MC – Sophia Children's Hospital. After retrieving (parental) informed consent, 321 patients with EA/TEF, admitted to the Department of Paediatric Surgery, were included. Pregnancy, clinical and follow-up data were extracted from medical charts. Available DNA and cell lines of patients ($n = 180$) and parents were collected and used for genetic analysis. Patients with a previous confirmed

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genetic syndrome, known chromosomal anomaly and/or pathogenic point mutation were excluded from further molecular–genetic evaluation. There is a weak evidence for an association of EA/TEF with certain environmental components;[7] however, we did not exclude any of the patients in our cohort based on these risk factors.

The database (>100 000 patients) of prenatal and postnatal diagnostics of our Department of Clinical Genetics was searched for triple X karyotypes and confirmed *SHOX* duplications.

Cytogenetic evaluation

Karyotyping was performed according to standard protocols on either lymphocytes from peripheral blood cultures or after amniocentesis. Karyotyping had been performed for 269 of the 321 patients of our cohort, as systematic cytogenetic follow-up of patients with congenital anomalies was not carried out before 1998.

MLPA and quantitative PCR

Not all array chips used had sufficient marker density in the PAR1 region. Therefore, we additionally screened for copy number variations in this region with MLPA, using the P036E1 and P070A2 Salsa Telomere Kit (MRC Holland, Amsterdam, The Netherlands) as described previously.[8] Genemarker 1.6 (SoftGenetics; LLC, State College, PA, USA) was used for data analysis. If duplications of the PAR1 region were detected, copy number profiling of the *SHOX* region was confirmed with a newly developed qPCR assay, with 11 amplicons within *SHOX* and the PAR1 region.[9]

Fluorescent *in situ* hybridization

The target BAC clone for Xp22 (RP11-800K15) and control probes on Xq25 (RP11-49N19) and 8p12 (RP11-489E7) were selected from the University of Santa Cruz (UCSC) genome browser (UC Santa Cruz, Santa Cruz, CA, USA; assembly March 2006) and ordered from BACPAC Resources (Children's Hospital of Oakland Research Institute, Oakland, CA, USA). After isolation of the BAC DNA, the probes were labeled and used for fluorescent *in situ* hybridization (FISH) on chromosome preparations from patients and parents, according to standard protocols.[10]

RNA-FISH analysis and immunocytochemistry of human cell lines

RNA-FISH and immunocytochemistry were performed on fibroblast cell lines (>90% confluence) and EBV-transformed lymphocytes, as previously described by Jonkers *et al*[11,12] using a 16.4 kb plasmid covering the complete *XIST* RNA sequence as described previously.[13,14]

HUMARA analysis

To determine the parental origin and the methylation status of the additional X chromosome, we used the HUMARA assay (human methylation of the androgen receptor assay) using 40 ng of genomic DNA input for the digestion reaction and gel electrophoresis to separate PCR products.[15]

Microarrays

The genome-wide copy number profile of all patients ($n=180$) was determined using either Affymetrix GeneChip Human Mapping 250K NSP1 (Affymetrix, Santa Clara, CA, USA), HumanQ610, HumanCytoSNP-12v1 to 2.1 or HumanOmniExpress (Illumina, San Diego, CA, USA). We generated Affymetrix CEL files with the Affymetrix genotype command console v.3.2 software (Affymetrix). The HumanCytoSNP-12v2.1 chip (Illumina) was used in the cases with *SHOX* duplications and their parents, for better coverage of the PAR1 region. All procedures were carried out according to the manufacturer's protocol as described previously.[16]

SALL1 mutation analysis

Sequencing of the coding region of the *SALL1* gene, including the splice sites, was carried out as described previously.[17] Primer sequences are available on request. We did not have sufficient DNA of triple X patient 3 to perform additional Sanger sequencing.

Table 1 Congenital anomalies of EA/TEF patients with X-chromosome anomaly

Pt. no.	*Karyotype*	*MLPA results*	*Maternal age*	*Clinical features*
1	47,XXX	X/Yp SHOX enh	25.9	Absent sacrum Anal atresia Pulmonary stenosis EA+TEF Vesicourethral reflux; urethral atresia Absent thumbs Cloacal malformation: abnormal labia, hydrometrocolpos
2	47,XXX	X/Yp SHOX enh	28.2	TEF Dysmorphic features
3	47,XXX	X/Yp SHOX enh	26.1	EA+TEF Ventricular septum defect Thin fingers
4	46,XY	X/Yp SHOX enh *paternal inheritance*	31.5	Aberrant subclavian artery EA+TEF Horseshoe kidneys Adducted thumbs; left thumb smaller than right Hypospadias Frontal bossing Dysmorphic features
5	46,XY	X/Yp SHOX enh *maternal inheritance*	34.3	Atrial septum defect (type II) EA+TEF Proximal placement of thumbs

Abbreviations: EA, esophageal atresia; enh, enhanced signal with MLPA kits P036E1 and P070A2; TEF, tracheoesophageal fistula.

Statistical analysis

Differences in two proportions were tested with the Pearson's χ^2-test, reported with a 95% confidence interval (CI), performed in SPSS 15.0 (IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

Patients included in the Erasmus MC – Sophia Children's Hospital EA/TEF cohort can be subdivided into four categories: isolated EA and/or TEF (45%), patients with one additional core VACTERL component (27%), VACTERL association, for example, three or more of the VACTERL core components (21%) and patients with a diagnosed genetic syndrome (7%).[18,19]

Triple X syndrome

A triple X karyotype (see Supplementary Figure 1) was identified in three EA/TEF patients, resulting in an odds ratio for triple X syndrome of 11.3 (95% CI = 3.6–35.2). All three were small for gestational age at birth (<5th percentile), with maternal ages ranging from almost 26 to 28 years. The first patient had all VACTERL features and additional genitourinary anomalies. The second patient had TEF and mild dysmorphic features and the third patient had EA/TEF, ventricular septal defect and thin fingers (patients 1–3; Table 1). SNP array analysis performed to exclude copy number variations elsewhere in the genome did not reveal additional possible pathogenic copy number variations in any of the three triple X patients.

Searching the database of our Department of Clinical Genetics yielded 59 non-mosaic 47,XXX karyotypes since 1988: 29 had been detected prenatally and 30 postnatally (apart from the above three patients). Indications (not mutually exclusive) for prenatal karyotyping were: maternal age >35 years ($n=25$), congenital malformations on ultrasound ($n=4$), increased risk for Down syndrome on first trimester screening ultrasound ($n=2$), increased echogenicity of the fetal bowel on ultrasound ($n=1$) and congenital anomalies in an earlier pregnancy ($n=3$). One screening, with an increased maternal age indication, concerned a twin pregnancy, of which one sib had a 47,XXX karyotype. Postnatal patients were karyotyped based on the following indications: suspicion of Fragile X syndrome ($n=5$), mental retardation ($n=5$), multiple congenital anomalies ($n=4$), combined mental retardation and multiple congenital anomalies ($n=3$), repeating spontaneous abortions in the index ($n=4$), failure to thrive ($n=4$), a chromosomal abnormality in the family ($n=3$), suspicion of trisomy 21 ($n=1$) and a possible chromosomal aberration in juvenile systemic lupus erythematosus ($n=1$).

Postnatal follow-up of 13 pregnancies was not documented. Seven pregnancies were terminated in one case because the fetus showed anencephaly. Congenital malformations had been documented for three of the nine births: a neural tube defect, hygroma colli with generalized edema and osteogenesis imperfecta, respectively. A review of the medical charts of the postnatally diagnosed patients identified at least four patients with congenital heart defects.

X-inactivation studies

Previous studies have indicated that only one X-chromosome remains active in somatic cells of 47,XXX patients. RNA-FISH and immunocytochemistry were performed to assess the X-inactivation status in cell lines derived from the 47,XXX patients of our cohort. RNA-FISH analysis revealed two *XIST* clouds in almost every fibroblast and lymphocyte cell (>95% of the nuclei), colocalizing with an area of low-level COT-1 expression (Figure 1a). Immunocytochemistry of 47,XXX fibroblasts detected enrichment of the facultative heterochromatin markers H3K27me3 and MacroH2A1 on two X-chromosomes, colocalizing with the DAPI-dense Barr bodies (Figure 1b). HUMARA analysis confirmed a maternal origin of the additional X chromosome in all three patients. In all these patients and their mothers random XCI was observed, with no skewed preference of inactivation of a particular X-chromosome (Supplementary Figure 3).

SHOX duplications

Enrichment of the triple X karyotype in our EA/TEF cohort prompted us to look further in our cohort for sex-chromosome aberrations with microarray and telomere-MLPA. These results indicated inherited *SHOX* duplications in two boys: patient 4 with a paternally inherited partial PAR1 duplication and patient 5 with a maternally inherited partial PAR1 duplication. Duplication of the *SHOX* gene is a rare event, it is only present twice in the database of genomic variation. Both *SHOX* duplications were confirmed with a *SHOX*-specific qPCR[9] (Figure 2a and Supplementary Figure 2).

To exclude additional potential pathogenic copy number variations in other regions in the genome of both patients and their parents, we analyzed their genome-wide copy number profile. These results revealed multiple copy number variations in both the patients and their parents. However, upon closer examination of these regions in the database of genomic variation, all of them were common polymorphisms. Given their relative high population frequency, they were not considered as potential pathogenic copy number variations in a relatively rare condition as EA/TEF or VACTERL association.

Patient 4's twin sib was spontaneously aborted in the third month of gestation. His mother was diagnosed with Goldenhar syndrome. She had right-sided hemifacial microsomia, anotia, deafness, paresis of the pallatum molle and facial dysmorphisms. Two relatives on the mother's side had thumb anomalies. EA/TEF or other major anomalies were absent in the father. The boy was small for gestational age at birth (<2 SD) and had associated cardiovascular, renal, limb and genital anomalies (Table 1).

SHOX-specific qPCR confirmed the duplication of the *SHOX* gene (amplicons 1–13) in the PAR1 region in patient 4 and his father. Cultured lymphocytes of proband or parents were not available for FISH validation or to localize the duplication. The 981 kb duplicated segment corresponds to the PAR1 region on chromosome X, and both *SHOX* variants, *SHOX*A g1-292dup and *SHOX*B g1-225dup, were completely duplicated in patient 4, including all of its regulatory sequences[20–22] (arr [hg18] Xp22 or Yp11(248 968–1 229 976) × 3) (Figures 2a and b and Supplementary Figure 4).

Patient 5, with apparently healthy parents, was also small for gestational age at birth and had cardiac and limb anomalies. He inherited a PAR1 duplication/suspected rearrangement from his mother overlapping the *SHOX* gene (arr [hg18] Xp22 (325 941–593 267) × 3) and separated by a region with normal copy numbers, a second Xp22 duplicated segment containing seven other genes (*IL3RA*, *SLC25A6*, *ASMTL*, *PP1164*, *P2RY8*, *SFRS17A* and *ASMT*) (arr [hg18] Xp22(1 428 051–1 891 174) × 3; Supplementary Figure 5). *SHOX*-specific qPCR (duplication of amplicons 2–8) confirmed the *SHOX* gene duplication in patient 5 and his mother. Moreover, FISH (BAC clone RP11-800K15) confirmed the location of the duplication at chromosome band Xp22 (see Supplementary Figure 6). The direction of the inserted duplicated segment was not determined.

SALL1 mutation analysis

Mutation analysis of the *SALL1* coding region only identified common variants, for example, two missense variants, one in

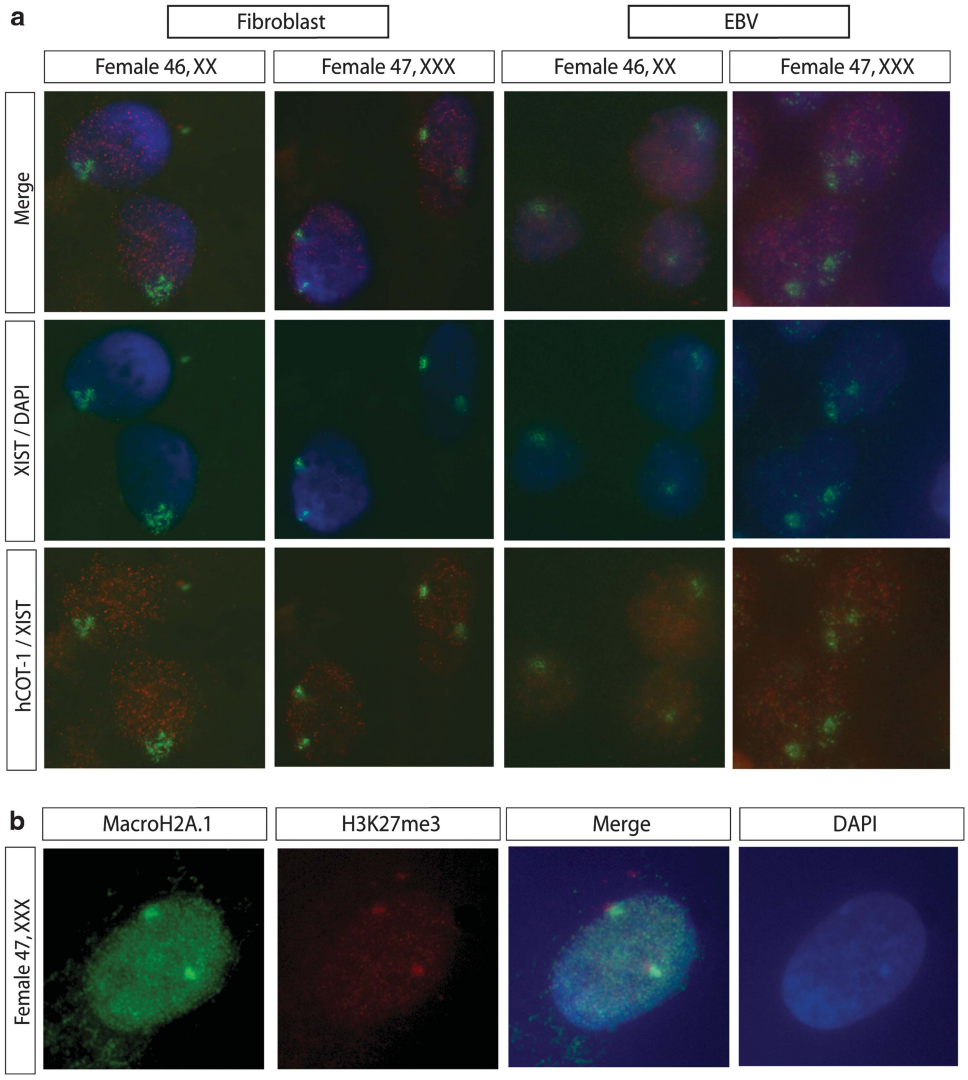

Figure 1 XCI studies in triple X patients. (**a**) RNA-FISH analysis detecting *XIST* and COT-1 RNA. *XIST* RNA was detected using a digeoxin-labeled cDNA probe[12,14] (fluorescein isothiocyanate (FITC), green), and a biotin-labeled COT-1 DNA probe detected expression of repetitive sequences (rhodamine, red). Characteristics for an inactive X-chromosome are the presence of *XIST* RNA accumulation and absence of COT-1 RNA. In 47,XXX patient cells, two *XIST* clouds and COT-1 holes were detected in the majority of the cells (>95% of the nuclei, $n>100$). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). (**b**) Immunocytochemistry detecting H3K27me3 (rhodamine, red) and MacroH2A1 (FITC, green). Enrichment of H3K27me3 and MacroH2A1 was found on two X chromosomes in 47,XXX fibroblast cell lines, colocalizing with DAPI-dense Barr bodies, indicative of two inactive X-chromosomes. Nuclei are counterstained with DAPI.

all patients and a control (rs4614723, minor allele frequency is 1.514%) and one in patient 4 (rs13336129, minor allele frequency is 6.651%). We also identified one intronic variant in patient 4 (rs13336129, minor allele frequency is 45.545%) and two synonymous variants in triple X patient 1 and a control (rs11645288, minor allele frequency is 17.816% and rs1965024, minor allele frequency is 49.216%).

DISCUSSION

Triple X syndrome and gastrointestinal anomalies

Congenital malformations and mental retardation syndromes have been linked to the X-chromosome. Our search for genes or loci involved in EA/TEF or other foregut-related anomalies identified five patients in our cohort with chromosome X/Yp aberrations: three with triple X syndrome and two with inherited PAR1 duplications.

A large proportion of triple X females have a subclinical phenotype; therefore, a triple X karyotype is usually a random finding in prenatal screening or cytogenetic follow-up of pregnancies. Most 47,XXX females remain undiagnosed; therefore, triple X syndrome has an estimated incidence rate of 0.10% with an average maternal age of 33.[4] Affected girls have a lower birth weight, more accelerated growth until puberty, long legs and an increase in behavioral problems and psychiatric disorder prevalence.[4] In 90% of patients, the additional X chromosome is the result of a maternal meiotic I error, and the incidence of non-disjunction errors increases with maternal age.[23]

Sex chromosome triploidies (47,XXX/47,XXY/47,XYY) are a rare (0.42%) finding in a large ($n=4282$) prenatal cohort analyzed with karyotyping and microarray.[24] Haverty *et al*[25] calculated the incidence rate in female subjects and found it to be 0.17%. In a recent European study of the EUROCAT working group, 0.054 triple X patients/1000 births were observed.[26] In our EA/TEF cohort, the incidence rate of

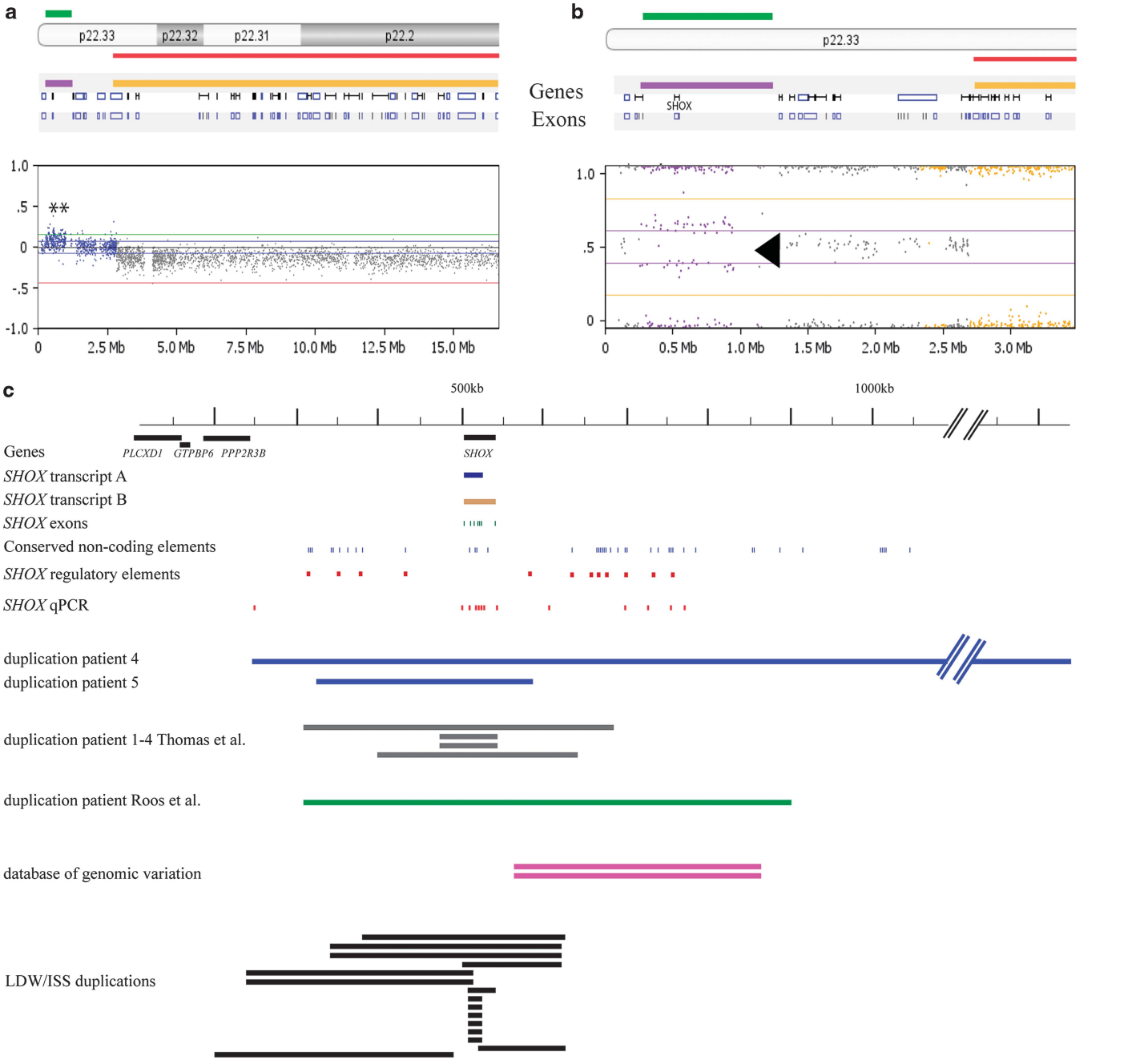

Figure 2 Triple X syndrome and SHOX aberrations in patients with esophageal atresia/tracheoesophageal fistula (OA/TOF). (**a**) The HumanCytoSNP-12 chip (Illumina) showing an interstitial duplication of the PAR1 region (base pair position chrX: 248 968–1 229 976), including the *SHOX* gene in patient 4. The elevated log 2 ratio indicates the duplicated segment (green bar). (**b**) The HumanCytoSNP-12 chip result visualized in Biodiscovery Nexus CN6.1. The B-allele frequency of patient 4 is indicated in the enlarged right panel (purple bar, arrow). The shift from a heterozygous state (gray dots, 0.5) to a 0.33/0.66 frequency (purple dots) is indicative for a copy number change. Taken together with the raise in the log R, this state is indicative for a copy number gain. (**c**) Schematic presentation of the SHOX A and B transcripts (dark blue and brown), the conserved noncoding elements (blue),[20] *SHOX* gene exons (green), SHOX qPCR (red)[9] and SHOX regulatory elements described in literature (dark red), the *SHOX* duplications in patients 4 and 5 and their position compared the duplications observed in patients 1–4 in Thomas *et al*,[49] Roos *et al*,[48] the database of genomic variation and the duplication in LWD/ISS patients described by Benito-Sanz *et al*.[43] The red lines indicate the length of the duplications, with a larger duplication in patient 4 (amplicons 1–13) and a *SHOX* duplication in patient 5 (amplicons 2–8) (Supplementary Figure 2). Both MLPA probes (orange) were duplicated in patients 4 and 5.

triple X syndrome is 1.12%, with an average maternal age of 30.8 years, which is 11 times higher than that in the estimated general population and 6.5 times higher than in the calculated incidence rate by Haverty *et al*.[25] Guichet *et al*[27] reviewed prenatally and postnatally diagnosed 47,XXX karyotypes from 18 laboratories. Mental retardation or congenital malformations were described in over one-third of the 190 patients reported. In all cases, weight-for-gestational-age at birth was under the 25th percentile. Congenital anomalies associated with the triple X syndrome described in case reports include anomalies of the urinary tract, genital anomalies and

craniofacial anomalies, especially a reduced head circumference and/or decreased brain volume.[4,28,29] Genitourinary malformations are well described in triple X patients. They often are associated with lower gastrointestinal tract anomalies; perhaps, a cloacal septation problem gives rise to the higher incidence of these types of malformations. Gastrointestinal anomalies, including atresia of the esophagus and duodenum and jejunum as well as omphalocele and anorectal malformations, have been reported sporadically.[6,25,30–36] All 13 reported patients with gastrointestinal and/or foregut-related anomalies are reviewed in Table 2. The patient described by Hoang *et al*[32] shows a similar phenotype compared to our patients: higher mesodermal defects (EA) and lower mesodermal defects (anal atresia, genitourinary defects).

X-inactivation patterns

The HUMARA assay demonstrated the maternal origin of the supernumerary X-chromosome and the absence of skewed preference for a particular X chromosome in our triple X patients. RNA-FISH analysis and immunocytochemistry demonstrated inactivation of two out of three X-chromosomes.

Overexpression of genes escaping X-inactivation could be responsible for the phenotypical abnormalities observed in our three EA/TEF patients and the gastrointestinal patients described in literature. Ten percent of genes have a variable pattern of gene X-inactivation and expression.[37] The extent of this escape is tissue specific, and often results in variable or lower levels of expression from the inactive X-chromosome compared with the active X-chromosome.[38,39] Why and how certain genes escape XCI, especially in humans, is still unknown.[40] Female 'escapees' may have a dosage-sensitive function, which would explain the phenotype in patients with sex chromosome anomalies, such as Turner and Klinefelter syndromes. The observation that 47,XXX females also have decreased brain volume in the presence of normal pubertal maturation suggests a possible direct dosage effect of X-chromosomal genes.[41] Two EA candidate genes *MID1* and *FANCB* do not escape X-inactivation, although there is a X-inactivation preference for the X chromosome that contains the mutated allele in Fanconi anemia.[24] Other genes that escape XCI could perhaps cause the gastrointestinal anomalies found in our cohort.

***SHOX* duplications**

One of those escapees, located in the PAR1 region, is *SHOX*. This gene has two isoforms: *SHOXA* and *SHOXB*, which are surrounded by several conserved noncoding regulators. [20] *SHOX* encodes a cell-specific homeodomain protein, and isoform A has an important role during human embryonic bone and limb development.[42] Two patients in our EA/TEF cohort have a duplication in the PAR1 region; the only overlapping duplicated gene is *SHOX*. The co-occurrence of *SHOX* duplication in a small cohort of a rare disease such as EA is intriguing, but we cannot exclude a chance finding. Large PAR1 duplications are relatively rare in the database of genomic variation (http://dgv.tcag.ca/), a database of 'healthy' individuals and only two duplications of *SHOX* are described. In the ISCA consortium patient database (http: // www.iscaconsortium.org/), duplications of *SHOX* are more prevalent (92 in total) and generally classified as uncertain.

However, we observed a *SHOX* duplication in two EA/TEF patients, and in both these patients limb development is disturbed. The limb and growth anomalies of the thump are different from the wrist deformity usually associated with *SHOX* deletions and duplications.[43,44] Furthermore, *SHOX* duplications are also associated with limb anomalies, for example, in Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome type 1[45], idiopathic short stature[46] or Leri–Weill dyschondrosteosis.[43]

The healthy carriers of the transmitted *SHOX* duplications in patients 4 and 5 seem to have the exact same duplication/rearrangement as their offspring. Absence of a phenotype in healthy parents could be caused by incomplete penetrance or variable expressivity as described for other microdeletion/duplication syndromes.[47] Roos *et al*[48] and Thomas *et al*[49] described five families with inherited *SHOX* duplications. The duplication was associated with cleft palate in two cases and one patient had a Madelung deformity. These and the *SHOX* duplications described by Benito-Sanz and co-workers[43,48,49] are often inherited from an unaffected parent. However, it is important to know the exact location of the *SHOX* duplication, as insertion of the duplicated segment could result in haploinsufficiency of *SHOX* by affecting the normal copy of the gene or its regulatory elements. It would certainly be beneficial to determine RNA and protein expression

Table 2 Congenital malformations of the gastrointestinal tract and/or foregut-related structures in triple X syndrome

N	Genital	Urinary	Gastrointestinal anomalies	V	A	C	TE	R	L	Other anomalies	Reference
3	+	+	Cloacal extrophy incl. imperforate anus, esophageal atresia + tef	+	+	+	+		+	Dysmorphic features	*Present study*
1			Jejunal atresia								34
1			Duodenal atresia								35
1			Duodenal atresia[a]			+					6
1			Omphalocele							Beckwith–Wiedemann syndrome	36
1			Omphalocele					+			31
1	+	+						+		Pulmonary hypoplasia, laryngeal atresia, craniofacial anomalies	56
1			Ectopic anus		+				+	Clinodactyly, inferior coloboma, clinodactyly, dysmorphic features	33
1	+	+	Cloacal extrophy incl. imperforate anus		+						25
1	+	+	Cloacal extrophy incl. imperforate anus and rectoperineal fistula, colonic atresia, omphalocele		+			+			30
1	+	+	Imperforate anus, esophageal atresia + TEF	+	+		+	+		Pulmonary hypoplasia, agenesis of gallbladder	32

Abbreviations: A, anorectal malformations; C, cardiovascular anomalies; GI, gastrointestinal malformations; L, limb malformations; N, number of patients; R, renal anomalies; TE, esophageal atresia and/or tracheoesophageal fistula; TEF, tracheoesophageal fistula; V, vertebral defects.
[a]Duodenal atresia due to annular pancreas.

in the esophageal or bone tissue; however, no biopsies of affected and corresponding normal tissues are available at this moment.

The mouse homolog of *SHOX* (*Shox2*) is involved in limb development,[50] and in humans *SHOX* enhancers are active in developing limbs.[51] *Sall1* and *Hoxd* mutant mice with limb anomalies quite similar to those observed in our patients have overexpression of Shox2.[52,53] *SALL1* is mutated in Townes–Brocks syndrome; patients suffering from this syndrome often have anal, renal and thump anomalies, and EA is a variable feature in this syndrome. Sequencing revealed no pathogenic *SALL1* mutations in the *SHOX* duplication patients.

We could speculate that *SHOX* duplication is the second hit in a two-hit model modulating, not causing, the abnormal development in these patients.

SHOX duplication and other rare inherited copy number variations could be modifying factors contributing to the broad phenotypical spectrum characteristic of the EA/TEF patient population.[19]

Genetic aberration (eg pathogenic mutations), aneuploidies and structural chromosomal changes like translocations, inversions or copy number variations have previously been detected in ~12.5% of patients in our cohort. This number will steadily increase, as it is expected that screening previously unresolved cases with whole exome sequencing or improved high-resolution microarray will identify both known and new causal genetic defects. Screening large patient cohorts for genetic defects can delineate new genetic syndromes when genotypes and phenotypes overlap, like recently published for the *EFTUD2* gene.[54]

In conclusion, we describe five patients with sex chromosomal aberrations and EA/TEF. All five patients had duplicated loci of pseudoautosomal genes, including *SHOX*, that escape X-inactivation and are candidates for a gene dosage effect. As a consequence of the additional X-chromosome, triple X female patients express more transcripts from genes that escape XCI. The expression of one or several of these genes could contribute to the phenotype. Overexpression of XCI escapees could shift the balance from normal to abnormal development in a small percentage of triple X patients. The expression levels of escaping genes on the inactive X-chromosomes may vary between individuals and different tissues.[55] As described previously, the phenotypic variability of triple X syndrome ranges widely from subclinical phenotypes to mental retardation and congenital malformations.[4]

The incidence of triple X syndrome in our EA/TEF cohort is 6.5–11 times higher than expected. Overexpression of XCI escapees, *SHOX* or other X-linked genes could be responsible for, or modulate, the phenotype of EA/TEF patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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