Microdeletion of 16q24.1–q24.2—A unique etiology of Lymphedema–Distichiasis syndrome and neurodevelopmental disorder

Marina Michelson1,2,3 | Gabriel Lidzbarsky4 | Daniella Nishri5 | Ifat Israel-Elgali3,6 | Rachel Berger2 | Michal Gafner3 | Noam Shomron3,6 | Dorit Lev1,2,3 | Yael Goldberg2,3,4

1Institute of Medical Genetics, Wolfson Medical Center, Holon, Israel
2The Genetic Institute of Maccabi Health Medicinal Organization, Tel-Aviv, Israel
3Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel
4Raphael Recanati Genetic Institute, Rabin Medical Center–Beilinson Hospital, Petach Tikva, Israel
5Child Developmental Center of Maccabi Health Medicinal Organization, Tel-Aviv, Israel
6Sagol School of Neuroscience, Tel-Aviv University, Tel-Aviv, Israel

Correspondence
Marina Michelson, Institute of Medical Genetics, Wolfson Medical Center, Holon, The Genetic Institute of Maccabi Health Medicinal Organization, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel.
Email: mashakerman@gmail.com

Abstract
Interstitial deletions of 16q24.1–q24.2 are associated with alveolar capillary dysplasia, congenital renal malformations, neurodevelopmental disorders, and congenital abnormalities. Lymphedema–Distichiasis syndrome (LDS; OMIM # 153400) is a dominant condition caused by heterozygous pathogenic variants in FOXC2. Usually, lymphedema and distichiasis occur in puberty or later on, and affected individuals typically achieve normal developmental milestones. Here, we describe a boy with congenital lymphedema, distichiasis, bilateral hydronephrosis, and global developmental delay, with a de novo microdeletion of 894 kb at 16q24.1–q24.2. This report extends the phenotype of both 16q24.1–q24.2 microdeletion syndrome and of LDS. Interestingly, the deletion involves only the 3′-UTR part of FOXC2.

KEYWORDS
16q24.1–q24.2 microdeletion, 3′-UTR FOXC2, congenital lymphedema, developmental delay, distichiasis

1 INTRODUCTION

Microdeletions of the long arm of chromosome 16 are not rare. In 1993, Callen et al. reported seven patients with interstitial deletions of 16q (Callen et al., 1993). Patients had global developmental delay, microcephaly, and dysmorphic features (Callen et al., 1993). The deleted segments comprised the interstitial parts of the 16q, occurring proximal to band 16q24.2. Since then, patients with haploinsufficiency of the 16q subtelomeric region have been identified with broad phenotypic variability (Handrigan et al., 2013; Kozlowska et al., 2020; Seeley et al., 2014; Stankiewicz et al., 2009; Szafrański et al., 2016; Szafrański et al., 2018; Yu et al., 2010; Zufferey et al., 2011).

Microdeletions at 16q24.2 are phenotypically apparent. Affected individuals present with intellectual disability, autistic spectrum disorder, seizures, speech delay and brain malformations, and congenital renal disease (Handrigan et al., 2013).

We describe a patient with a deletion at 16q24.1–q24.2 who presented with congenital lymphedema, distichiasis, developmental delay, and congenital hydronephrosis. Lymphedema–Distichiasis syndrome (LDS) is a distinct condition caused by heterozygous pathogenic variants in FOXC2. LDS may also be associated with renal disease and diabetes mellitus (Yıldırım-Toruner et al., 2014).

The deleted region harbors the morbid gene FBXO31 and the 3′-UTR region of FOXC2.
Although FOXC2 and FBXO31 have been included in some of the reported cases, LDS has not been reported as part of the syndrome. We compare the features of our patient with the reported 16q24 microdeletion syndrome cases and to those described with LDS.

2 | MATERIALS AND METHODS

2.1 | Chromosomal microarray analysis

Deoxyribonucleic acid (DNA) extraction from peripheral blood was performed by the MagNA Pure Compact (MPC) nucleic acid isolation kit I and an Automated MPC instrument (Roche Diagnostics) in accordance with manufacturer’s protocol. Quantity and quality assessment of the extracted DNA was performed by a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.). DNA samples were diluted to a concentration of 50 ng/ml.

Chromosomal microarray (CMA) analysis was performed using Illumina Human Omni express (GxG Comprehensive Array, v1.0 Beadchip 709,671 SNP loci) microarrays. Gene by Gene’s GxG Comprehensive Array analysis was done. Coordinated are according to UCSC Genome Browser GRCh37.

2.2 | Expression studies

Expression analysis was done on the proband and his healthy parents. Ribonucleic acid (RNA) was extracted from peripheral blood mononuclear cells (PBMC) using TRIzol reagent (Thermo Fisher Scientific). Reverse transcription reactions for mRNA were performed using the High-Capacity cDNA Reverse-Transcription Kit with random primers, according to the manufacturer’s protocol (Thermo Fisher Scientific). Real-time quantitative PCR (RT-PCR) was performed using Quanta qPCR Gene Expression Master Mix (Quanta Technology). Comparative critical threshold (Ct) values, obtained by real-time PCR analysis, were used for relative quantification of gene expression, and determination of the fold-change of expression. Fold change values were obtained using the formula: $\Delta \Delta Ct$ (Schmittgen & Livak, 2000). Normalization for mRNAs was performed compared to human B-actin expression. Primers sequences: Forward-AGCACAAACTTTTCCCAACG, Reverse-CATTGCCACTCA CCTGGGA.

2.3 | Sanger cDNA sequencing of FOXC2

In order to confirm that the proband possesses a wild type copy of the FOXC2 3’-UTR, a region located downstream of the deletion was amplified (using PCR) and sequenced. Complementary DNA (cDNA) was synthesized, and amplification of FOXC2 was performed using custom primers: 5’-ATTCTTCCAACCGTGTCTAC-3’, 5’-ACTTATCCAGTG AACTCAACTT-3’. The PCR product was run through a 1.5% Agarose gel. Discrete bands were extracted from the gel and were confirmed using Sanger sequencing technology.

3 | RESULTS

3.1 | Clinical characterization

The clinical features of the affected patient are summarized in Table 1. The proband is a 6-year-old boy who first attended the genetic clinic at 10 months of age due to congenital lymphedema, hypotonia, and global developmental delay.

The boy is a son of healthy nonconsanguineous parents of Bukharin Jewish origin. Family history is negative for neurological disorders or congenital anomalies.

The pregnancy was uneventful. Fetal sonographic scan revealed bilateral pyelectasis. He was born at term; birth weight and occipital frontal circumference were within normal ranges. He was diagnosed with moderate hydronephrosis and vesicourethral reflux.

During the first weeks of life, moderate swelling of both calves and feet, more on the right leg, occurred. Ultrasound of lower extremities revealed increased skin and subcutaneous thickness, and pronounced subcutaneous echogenicity, with normal venous Doppler ultrasound.

His development was slow without stagnation or regression. He walked independently at 19 months. Single words appeared at 13 months of age; however, further attainment was significantly delayed. He attained special education since 3 years of age. At that age, his vocabulary and understanding were significantly limited. He was diagnosed with attention deficit hyperactivity disorder at the age of 5 years.

At the age of 5 years, physical development and head circumference were age appropriate. The right foot was longer than the left. There was a moderate difference in the lower shin circumference (right thicker than left; Figure 1b).

Distichiasis-double rows of eyelashes was observed at the age of 6 years (Figure 1a).

3.2 | Chromosomal microarray

CMA showed a 894.4 kb deletion at genomic coordinates chr16:86602575–87497027 (GRCh37; Figure 2). The proband also had a maternally inherited 317 kb duplication at chr8:14779676–15096705, classified as likely benign. The 16q24 deletion was de novo. It and included the OMIM morbid genes, which are not associated with diseases.

Full details of the genes located in the deletion can be found in Table S1.

This deletion detected for the first time in our cohort of 53,498 CMA cases done in Maccabi HMO from August 2014 to December 2020, of them 6195 postnatal tests, done on patients with intellectual deficiency, ASD or major malformations. Sanger sequencing of FBXO31 did not detect any suspected variant.

3.3 | Real-time PCR

Real-time PCR analysis of FOXC2 expression levels demonstrated 71% reduction of expression in PBMCs from the proband, compared to PBMCs from control samples ($p = 0.031$; Figure 3).
| Described by | Present case | Stankiewicz et al. | Yu S et al. | Zufferey et al. | Garabedian et al. 2012 | Szafranski et al. | Kożłowska et al |
|-------------|--------------|--------------------|-------------|-----------------|-----------------------|-------------------|-----------------|
| **Number of patients** | 1 | 10 | 1 | 1 | 1 | 13 | 2 |
| **Deleted region** | 16q24.1–q24.2 | 16q24.1–q24.2 | 16q24.1–q24.2 | 16q24.1 | 16q24.1 | 16q24.1 | 16q24.1 |
| **Genome coordinates (GRCh37/hg19)** | 86602575–87497027 | chr16:85890261–7257585 | chr16:85890261–7257585 | chr16:85890261–7257585 | chr16:85890261–7257585 | chr16:85890261–7257585 | chr16:85890261–7257585 |
| **Mode of inheritance** | De novo | De novo (8/9) Maternal (1/9) | De novo | NA | NA | De novo 12/13 | NA |
| **OMIM MORBID genes** | FOXC2 3′-UTR | FOXC2 (8/10) FOXF1 (6/10) | FOXC2 | FOXC2 FOXF1 IRF8 | FOXC2 FOXC2 FOXF1, IRF8 COX4I1 COX4N1 | FOXC2 FOXF1 | FOXC2 FOXF1 |
| **Other deleted genes** | MAP1LC3B FOXL1 ZCCHC14 | MTHFSD FOXL1 (5/10) | MTHFSD FOXL1 | MTHFSD FOXL1 | MTHFSD FOXL1 | MTHFSD FOXL1 | MTHFSD |
| **NDD disorder** | Global developmental delay | Developmental delay (Bell et al., 2001) Ventriculomegaly, Chiari malformation. NA (Jin et al., 2020) due to early death | No | No | No | None | None |
| **Genitourinary anomalies** | Congenital hydronephrosis | 6/10: Hydronephrosis, uretero-pelvicaliectasias | Hypospadias, hydronephrosis tortuous dilated ureters, urethral obstruction | Pelvicaliectasis with ureteral stenosis | No | Renal agenesis | 1/2: Hydronephrosis |
| **Lymphedema** | Congenital lymphedema | None | No | No | No | None | None |
| **Distichiasis** | Yes | None | No | No | None | None | None |
| **Multiple congenital anomalies** | No | ACDMPV (5/10) CHD: (6/10): hypoplasia of left ventricle TOF, VSD Gastrointestinal Malformations: (5/10): tracheoesophageal fistula, esophageal atresia, duodenal and anal atresias, imperforate anus Single umbilical artery (3/10) Cleft lip and palate (1/10) Butterfly vertebrae (2/10) | ACDMPV: hypoplasia of left ventricle, pulmonary valve atresia, subaortic VSD with overriding of aorta, pulmonary artery stenosis, patent foramen ovale, persistent left superior vena cava; Intestinal malrotation | ACDMPV: CHD: AV canal, dysplastic tricuspid and mitral valve; Annular pancreas, duodenal dilatation | Cystic hygroma; Fetal hydrops; Single umbilical artery | ACDMPV; CHD; Esophageal fistula, gut malrotation, absent gall bladder; imperforate anus, single umbilical artery | 1/2: Polyhydramnion omphalocele |

Abbreviations: ACDMPV, alveolar capillary dysplasia with misalignment of pulmonary veins; ADHD, attention deficit hyperactivity disorder; CHD, congenital heart disease; NA, not available; NDD, neurodevelopmental disorders; TOF, tetralogy of Fallot; VSD, ventricular septal defect.
3.4 | Sanger cDNA sequencing of FOXC2

The sequencing matched the FOXC2 mRNA sequence (Figure S1), implying the presence of a 3'-UTR from the wild-type copy of the FOXC2 gene for the proband.

4 | DISCUSSION

The overlapping deletions at 16q24.1–q24.2 have been described with diverse associations. These included alveolar capillary dysplasia, cystic hygroma and hydrops fetalis, structural brain malformations, unspecific dysmorphic features, autism, and vascular malformations (Table 1).

We describe a patient with 16q24.1–q24.2 microdeletion with congenital lymphedema, distichiasis, developmental delay, and renal abnormalities.

Previous studies have shown that microdeletion at the 16q24.1–q24.2 may be associated with neurodevelopmental disorders (Table 1).

However, the deletion in our patient is proximal to those reported cases. The overlapping deleted region included the OMIM morbid FBXO31 and four other genes—ZCCHC14, MAP1LC3B, FOXL1, and C16orf95 (Table 2), which scarce information regarding neurodevelopment exists about them.

FBXO31 controls neuronal morphogenesis and migration in the developing brain (Vadhvani et al., 2013). Bi-allelic mutations in that gene have been associated with intellectual disability (Mir et al., 2014). Sequencing of the gene did not detect additional pathogenic variant. Recently, two patients with cerebral palsy, heterozygous for de novo mutations in FBXO31 were described (Mental retardation autosomal recessive-45, OMIM # 615979; Jin et al., 2020). Therefore, FBXO31 may contribute to the neurodevelopmental delay, either
directly or by an effect on the allelic architecture (Yuan et al., 2020). The deleted region in our patient also includes long noncoding RNAs that may affect the phenotype. Recent studies have demonstrated the role of long noncoding RNAs in CNS development, by regulation of gene expression in neuronal differentiation, synaptogenesis, and synaptic plasticity (Cuevas-Diaz Duran et al., 2019).

LDS is a distinct syndrome characterized by unique combination of lymphedema and distichiasis (McDermott & Lahiff, 2016). The lymphedema is confined to the lower limbs and appears in puberty or later on. Distichiasis usually occurs in puberty or in young adulthood (Table 1).

**TABLE 2** Clinical features in current patient and previously reported patients with FOXC2 variants

| Described by              | Presented case | Bell et al. 2001 | Erickson et al. 2001 | Finegold et al. 2001 | Brice et al. 2002 | van Steensel et al. 2001 | Tavian et al. 2016 | Wallis et al. 2021 |
|---------------------------|----------------|------------------|----------------------|---------------------|-------------------|-------------------------|--------------------|-------------------|
| Number of patients        | 1              | 14               | 31                   | 44                  | 74                | 11                      | 6                  | 5                 |
| Mutation                  | 3′-UTR deletion| Frameshift       | Truncating           | Truncating          | Frameshift, Missense | Missense               | Frameshift, Stop codon | FOXC2 promoter- enhancer dissociation due to balanced translocation t (16;22) (q24; q13.1) |
| Lymphedema – age of onset | Birth          | Puberty or later on | 4–82 yo             | 6–80 yo 2 cases-birth | 11–36 yo         | 6–16 yo                 | 14–50 yo           | 15 yo             |
| Distichiasis – age of onset| 6 yo          | Puberty          | puberty              | 0–30 yo            | puberty           | 2/11                    | NA                 | 26–48 yo NA       |
| Renal anomalies           | Bilateral      | Hydro nephrosis  | None                 | None               | None             | 5/74                    | None               | None              |
| NDD disorder              | Global         | Developmental    | None                 | None               | None             | 1/4 learning disabilities and autistic features | None               | None              |
| Other anomalies           | No             | Varicose veins CHD Pierre Robin sequence Scoliosis | 2/31 Cystic hygroma TOF Cleft palate | 1/44 Cystic hygroma, TOF Cleft palate Yellow nail | Varicose vein CHD Scoliosis | Varicose vein CHD Scoliosis | 1/6 Bicuspid aortic valve | Hydrops Nuchal edema |

Abbreviations: ADHD, attention deficit hyperactivity disorder; CHD, Congenital heart disease; NDD, neurodevelopmental disorders; TOF, tetralogy of Fallot; Yo, years old.

Forkhead transcription factor (FOXC2) is considered the only causative gene for LDS (Tavian et al., 2016; van Steensel et al., 2009). FOXC2 regulates genes and signaling pathways involved in lymphangiogenesis (Norden et al., 2020; Wu & Liu, 2011). Mutations impair transcriptional activity and cell proliferation (Tavian et al., 2020). FOXC2 also negatively regulates increased Ras/ERK signaling during lymphangiogenesis.

LDS phenotype is caused by numerous mutations along the entire gene, and has been attributed to promoter-enhancer dissociation of a topological-associated domain (Wallis et al., 2021; Table 2). The CMA results, presented here, include only the 3′-UTR of the FOXC2 gene. 3′-UTR regulates translation efficiency of synthesized protein, mRNA stability, export to cytoplasm, and subcellular localization (Matoulkova et al., 2012). rs1035550 (NM_002521:3:c.*260A>C/T/G), a variant in the FOXC2 3′-UTR, was associated with secondary lymphedema following breast cancer surgery (Miaskowski et al., 2013) and risk of varicose veins (Shadrina et al., 2016). The FOXC2 3′-UTR also contains several MicroRNAs (miRNA) targets (Nimir et al., 2017). MiRNAs were found to play a role in embryonic lymphangiogenesis through the activation the NFATC1 transcriptional factor, which is associated with FOXC2. Knock down of endothelial miRNAs have shown to result in defective lymphatic vessels development (Jung et al., 2019). Regulation of gene expression through 3′-UTR was shown to be directly mediated by overexpression of miR-204 and miR-495, and affected by miR-374c-5p and MiR-204-5p (Yang et al., 2017).

The role of the FOXC2 3′-UTR deletion is further supported by the results of the current study. Quantitative PCR results showed significantly lower expression level of FOXC2 in the proband compared to his parents, thus supporting the genomic finding. To support a
possible role of heterozygote deletion in the FOXC2 3′-UTR, this region was sequenced using DNA and cDNA samples. Both tests showed the existence of wild-type allele.

In conclusion, we report a novel phenotype of 16q24.1–q24.2 microdeletion syndrome of congenital LDS. Our results indicate a possible newly described role of FOXC2 3′-UTR deletion in LDS, which needs to be further studied.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
The authors confirm contribution to the paper as follows: study conception and design: Marina Michelson, Yael Goldberg, Dorit Lev; data collection: Marina Michelson, Daniella Nishri, Rachel Berger, Noam Shomron, Ifat Israel-Elgali; data analysis and interpretation: Marina Michelson, Gabriel Lidzbarsky, Yael Goldberg, Ifat Israel-Elgali; draft manuscript preparation: Marina Michelson, Gabriel Lidzbarsky, Michal Gafner; critical revision of the article: Yael Goldberg, Dorit Lev. All the authors reviewed the results and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID
Marina Michelson https://orcid.org/0000-0002-3794-0843
Michal Gafner https://orcid.org/0000-0002-3851-7334
Dorit Lev https://orcid.org/0000-0001-6869-6727

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SUPPORTING INFORMATION

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