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Comprehensive Genetic Analysis of OEIS Complex Reveals No Evidence for a Recurrent Microdeletion or Duplication

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Omphalocele–exstrophy of the bladder-imperforate anus-spinal defects (OEIS) complex, or cloacal exstrophy (EC), is a rare constellation of malformations in humans involving the urogenital, gastrointestinal, and skeletal systems, and less commonly the central nervous system. Although OEIS complex is well-recognized in the clinical setting, there remains a significant lack of understanding of this condition at both the developmental and the genetic level. While most cases are sporadic, familial cases have been reported, suggesting that one or more specific genes may play a significant role in this condition. Several developmental mechanisms have been proposed to explain the etiology of OEIS complex, and it is generally considered to be a defect early in caudal mesoderm development and ventral body wall closure. The goal of this study was to identify genetic aberrations in 13 patients with OEIS/EC using a combination of candidate gene analysis and microarray studies. Analysis of 14 candidate genes in combination with either high resolution SNP or oligonucleotide microarray did not reveal any disease-causing mutations, although novel variants were identified in five patients. To our knowledge, this is the most comprehensive genetic analysis of patients with OEIS complex to date. We conclude that OEIS is a complex disorder from an etiological perspective, likely involving a combination of genetic and environmental predispositions. Based on our data, OEIS complex is unlikely to be caused by a recurrent chromosomal aberration.

Key words: omphalocele–exstrophy of the bladder-imperforate anus-spinal defects (OEIS) complex; cloacal exstrophy; candidate gene analysis; array CGH

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INTRODUCTION

Omphalocele–exstrophy of the bladder-imperforate anus-spinal defects (OEIS) complex or cloacal exstrophy (EC), is a rare constellation of malformations involving multiple organ systems in humans [Carey et al., 1978; Kallen et al., 2000; Keppler-Noreuil, 2001; Martinez-Frias et al., 2001]. The incidence of OEIS complex is estimated to be 1 in 200,000 live births although this may be an underestimate of the total number of cases because it lacks inclusion of stillbirths and pregnancy terminations [Martinez-Frias et al., 2001; Keppler-Noreuil et al., 2007]. Nevertheless, individuals born with this malformation complex require immediate

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surgical intervention and suffer from significant long-term disabilities.

The pathophysiology of OEIS complex is unknown. OEIS/EC is hypothesized to be part of a spectrum of malformations, also known as BEEC, which includes bladder exstrophy (BE) and epispadias. Several hypotheses have been proposed to explain the developmental mechanisms leading to the phenotypic expression of OEIS complex and BE [Keppler-Noreuil, 2001; Martinez-Frias et al., 2001; Bohring, 2002; Siebert et al., 2005; Sadler and Feldkamp, 2008; Ludwig et al., 2009a]. OEIS complex is thought to be caused by a defect in caudal mesoderm formation and migration between the ectodermal and endodermal layers of the cloacal membrane, leading to premature or abnormal rupture of the cloacal membrane. The phenotype of OEIS complex versus BE is hypothesized to be due to the timing of abnormal cloacal membrane rupture with respect to migration of the urorectal septum [Jones, 1997; Keppler-Noreuil, 2001]. More recently, it has been proposed that OEIS/EC is due to failure in closure of the ventral body wall, which occurs during the third and fourth weeks of development [Sadler and Feldkamp, 2008]. Limb-body wall complex (LBWC) and urorectal septum malformation sequence (URSMS) also share overlapping features with OEIS/EC, and it has been hypothesized that they also share an underlying etiology [Heyroth-Griffis et al., 2007]. Other malformations of the caudal portion of the embryo share some features with OEIS complex, including vertebral-anal -cardiac-tracheoesophageal fistula-renal-limb (VACTERL) association and caudal regression syndrome (CRS) [Bohring, 2002; Siebert et al., 2005]. All of these conditions have an unknown etiology.

Most cases of OEIS complex are sporadic. However, reports of familial cases and higher rates of concordance in monozygotic twins provide support for a genetic etiology [Lee et al., 1999; Keppler-Noreuil, 2001; Boyadjiev et al., 2004]. Although rare chromosomal anomalies have been reported and a few candidate genes have been analyzed in small numbers of patients [Nye et al., 2000; Boyadjiev et al., 2004, 2005; Reutter et al., 2006; Kruger et al., 2008; El-Hattab et al., 2010], no causative genes have been identified. Environmental influences have also been proposed, although no clear environmental predisposition has emerged from epidemiological studies [Kallen et al., 2000; Martinez-Frias et al., 2001; Boyadjiev et al., 2004; Keppler-Noreuil et al., 2007].

Here, we report on a genetic analysis of 13 individuals with OEIS complex. For this analysis we considered that it was important to analyze only OEIS cases and not isolated BE. To identify pathogenic copy number variations (CNVs) that might be responsible for the OEIS phenotype, we performed high resolution SNP and oligonucleotide microarrays on our patient cohort. We also sequenced 14 candidate genes that were predicted to cause an OEIS complex phenotype in humans based on caudal and/or urogenital defects in humans or in mouse models and/or expression patterns. Although no pathogenic mutations were identified in our patient sample, this is the most comprehensive genetic analysis of patients with OEIS complex to date. Based on our data, we can conclude that OEIS complex is unlikely to be caused by a recurrent chromosomal microdeletion or duplication.

MATERIALS AND METHODS

Experimental Subjects

Written informed consent was obtained from all study participants in accordance with approved protocols from the Institutional Review Board of the University of Michigan. Clinical features of the 13 patients included in this study are shown in Table I. All patients had at least two of the cardinal features of OEIS complex, with the majority having all four cardinal features. One sample (Patient 19) was from a fetus with the characteristic ultrasound findings of OEIS complex. DNA was isolated from blood or buccal samples using the Gentra Puregene DNA isolation kit (Qiagen-USA, Valencia, CA) per manufacturer instructions. Buccal DNA samples were obtained only from parents for confirmatory testing.

PCR and DNA Sequencing of Candidate Genes

PCR was performed in 20 µl reaction volumes containing 1 µM forward and reverse primers and 1× Taq-Pro Complete (Denville Scientific, Metuchen, NJ). PCR cycling was performed in an Eppendorf Mastercycler (Eppendorf North America, Westbury, NY) with conditions as follows: initial denature 95°C for 15 min, 25 cycles 95°C for 30 sec, 72°C −0.7°C/cycle for 30 sec, 72°C for 1 min, 21 cycles 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, followed by a single 72°C extension for 10 min. PCR primer sequences are available by contacting the corresponding author. PCR results were analyzed on 1% agarose gels electrophoresed in 1× Tris-acetate buffer. PCR products were purified using Qiagen PCR purification columns (Qiagen-USA) and sequenced by the University of Michigan DNA sequencing core. Sequence analysis was performed by aligning patient DNA sequences to the human genome consensus sequence (hg18/build 36, released March 2006 as viewed at http://www.genome.ucsc.edu) using the Sequencher computer program (GeneCodes, Ann Arbor, MI). All DNA changes identified were compared to known polymorphisms located in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). Changes identified as novel were tested in parental DNA samples when available.

Mutation Analysis of the MT-RNR1 Gene

Mutation screening of the mitochondrial chromosome at position 1555 within the *MT-RNR1* gene was performed by PCR and RFLP analysis as previously described [Nye et al., 2000].

TaqMan Assays

TaqMan DNA copy number assays were performed on an ABI7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions were performed in 25 µl volumes containing 1× FAM-labeled locus specific TaqMan gene copy assay, 1× VIC-labeled *RNase P* copy number reference assay, 1× TaqMan gene expression master mix, and 40 ng template DNA. Cycling conditions were hold/denature 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec. The manual C_T threshold of the ABI7500 was set to 0.2, and the autobaseline was on. Taqman assay Hs04956436_cn representing the *CHN2* gene and assay Hs00345272_cn representing the *ANKYF1* gene were used for patients number 1 and

TABLE I. Clinical Findings of Patients Included in Genetic Analysis of OEIS Complex

	Omphalocele	Bladder exstrophy	Spinal defect	Imperforate anus	Vertebral anomalies	Other GI defect	Other genitourinary defect	Limb defect	CV defect	CNS defect	Lung defect	No. cardinal features of OEIS	Outcome
Patient 1	+	+	+ (lipomyelocystocele)	+	Partial SA	NI	Epispadias, bifid phallus, testes palpable in scrotum, small kidneys	Club feet	Secundum ASD	Cyst in R medulla	Tracheobronchomalacia	4/4	Died age 2 months
Patient 2	+	+	+ (TC)	+	UNK	NI	Absent uterus and cervix; single kidney	NI	NI	NI	NI	4/4	Living 20 years
Patient 4	+	+	+ (TC + lipoma)	+	UNK	UNK	Small R pelvic kidney	NI	NI	NI	NI	4/4	Living 18 years
Patient 5	No	+ (bilateral hemi-bladders)	+ (myelocystocele + TC)	+	T10, T11, partial SA	NI	Gonad present in R labial-scrotal fold; small L kidney	NI	NI	NI	NI	3/4	Living 17 years
Patient 6	+	+	+ (terminal myelocystocele + syrinx and TC)	+	NI	NI	NI female genitalia; small dysplastic R kidney	NI	PDA	NI	NI	4/4	Living 7 years
Patient 9	+	+	No	+	NI	Recto-vaginal fistula	Recto-vaginal fistula	Vascular malformation of L leg	NI	NI	NI	3/4	Living 4 years
Patient 11	+	+	+ (TC)	+	T5–T8, partial SA	NI	Vaginal duplication; R hydronephrosis	Club feet	PDA; hemiazygous vein	NI	NI	4/4	Living 4 years
Patient 13	+	+	+ (terminal myelocystocele + TC)	+	T7, multiple lumbar, partial SA	Pro-lapsed ileum	Epispadias, bifid phallus, undescended testes, small R kidney with megaureter	Club feet	PFO	Type I Chiari	NI	4/4	Living 3 years
Patient 14	+	+	+ (lipomyelocele + syrinx)	+	Dysraphic hypoplastic sacrum, pubic diastasis	Pyloric stenosis	Bicornuate uterus, R hydronephrosis, hydroureter	L metatarsus varus	PDA, PFO vs. ASD, PV and TV regurg.	NI	NI	4/4	Living 3 years
Patient 15	+	+	+ (TC)	+	T8, L4–L5	NI	Epispadias, L testis in scrotum, R inguinal testis	NI	NI	UNK	NI	4/4	Living 3 years
Patient 16	+	No	+ (R lateral myelomeningocele + syrinx and TC)	No	T10, T11–12, fusion ribs R 8–9, absent ribs R 11–12	Missing R abd musculature	Asym of labia majora, NI renal U/S	NI	NI	NI	Pulmonary hypoplasia	2/4	Living 4 years
Patient 17	+	+	+	+	UNK	UNK	"Ambiguous genitalia," NI renal U/S	Club feet	PDA/PFO	UNK	Pulmonary hypoplasia	4/4	Living 2 years
Patient 19 (fetus)	Ventral wall defect	Bladder not identified	Probable myelomeningocele	UNK	Sacral vertebral bodies splayed	UNK	Prominent renal pelvis	Appear NI	UNK	NI	UNK	3/4	Selective reduction (twin preg.) ~16 weeks

TC, tethered cord; SA, sacral agenesis; NI, normal; UNK; unknown, +, present; R, right; L, left.

number 9 respectively. A control assay (Hs05018555_cn) was also performed to detect a deletion carried by patient 11 mapping to chromosome 7p21.3. All reactions were performed at least two times in triplicate. DNA copy number was calculated using Applied Biosystem's CopyCaller software version 1.0 comparing locus specific copy number to the *RNase P* control.

SNP Chip Analysis

DNA for microarray analysis was purified over a QIAamp spin column (Qiagen, QIAGEN Benelux B.V., KJ Venlo, the Netherlands) followed by digestion with *Nsp*I. Specific adaptors were ligated and fragments of 200–1,000 bp were amplified via optimized PCR conditions. The amplified DNA was then fragmented, labeled, and hybridized to a GeneChip Mapping 250K SNP Array (Affymetrix, Santa Clara, CA). The 250K SNP microarray was run on the GeneChip Scannner 3000 7G with autoloader, GeneChip Fluidics station 450, and GeneChip Hybridisaton oven 640 (Affymetrix).

The 250K SNP data were analyzed with GeneChip Genotyping Analysis Software (GTYPE, Affymetrix), which uses an automated, model-based genotype-calling algorithm that provides a confidence score for each individual genotype. Copy number estimates were determined automatically using the CNAG 2.0 software package by a hidden markov model [Nannya et al., 2005]. Microdeletions and duplications detected by SNP microarray analysis were first compared to an in-house reference dataset of patients with disorders other than OEIS complex as well as to the Database of Genomic Variants (http://projects.tag.ca/variation). Copy number changes that occur as non-pathogenic variants in the human genome were excluded from further analysis.

Oligo Array Analysis

In separate tubes, 1,010 ng of patient genomic DNA and of sex-mismatched pooled reference genomic DNA were double-digested with the restriction enzymes *Alu*I and *Rsa*I (Promega, Madison, WA) and then fluorescently labeled with Cy3 and Cy5 respectively, using the Genomic DNA Labeling Kit Plus (Agilent Technologies, Santa Clara, CA). Labeled DNA samples were cleaned up of reagents and unincorporated dyes by vacuum filtration using the Multi-Screen HTS Filtration plates (Millipore, Billerica, MA) and re-suspended in 22 µl of 1× tris-EDTA. Purified fluorescently labeled patient and reference DNAs were mixed together, and hybridized to an Agilent 244K oligonucleotide array using standard reagents and protocols supplied by the array manufacturer (Agilent Technologies). Arrays were hybridized for 40 ±4 hr, and washed using Agilent wash procedure B.

Arrays were scanned on a GenePix 4200A scanner (Molecular Devices, Sunnyvale, CA), enclosed in a NoZone® TL Workspace, using GenePix Pro 6.1 software. Gains and losses were called according to interpretation criteria established by the International Standard Cytogenomic Array (ISCA) Consortium, as described in Baldwin et al. [2008]. The resulting log2 derivatives of the patient/control signal intensity ratios were plotted relative to the genomic location of the corresponding probes. For detection of gains or losses the thresholds for the log2 ratios were set at −0.32 for losses and 0.26 for gains. A minimum of 4 contiguous oligonucleotide probes showing loss or gain of signal intensity is needed to make an aberration call.

RESULTS

SNP Chip Analysis

Affymetrix 250K SNP arrays were run on seven patient samples from our cohort. DNA gains and/or losses were detected in four patient samples tested, and a total of 11 different changes were identified (Table II). Ten of the changes were unique, while one occurred in three of the seven samples tested. The recurrent change is a duplication mapping to chromosome 17q21.31 covering bases 41,521,621–41,647,903 (March 2006 human genome build (hg18) as displayed on the UCSC Genome Browser). The DNA that is duplicated in our patients flanks a known disease locus. Deletion

TABLE II. Copy Number Variation Identified by SNP Array

Patient	Chromosome band	Location	Type	Change
#1	7p15.1	29165987–29510059	Gain	+1
	17q21.31–q21.32	41513416–42150418	Gain	+1
#2	5q21.1	101110353–101122504	Gain	+1
	11p15.1	18907033–18918255	Gain	+1
	17q21.31–q21.32	41521621–41647903	Gain	+1
	22q11.1	15268818–15659603	Gain	+1
	Xp22.31	7355904–7413831	Loss	−1
#9	17p13.2	3945948–4270085	Gain	+1
	18q12.1	27230880–27295732	Gain	+1
	4p15.31	18595704–18733330	Loss	−1
	6q21	110348917–110400167	Loss	−1
#11	7p21.3	8735273–8961255	Loss	−1
	17q21.31–q21.32	41474846–41707706	Gain	+1

Copy number changes were not identified in patients #4, #5, and #6.

of the *MAPT* locus on chromosome 17q21.31 is a known cause of a syndrome characterized by mental retardation and dysmorphic features [Koolen et al., 2006]. However, the region we identified as duplicated in our patient cohort is distal to this known genomic disorder, is reported to be extremely polymorphic for copy number, and is well represented in the database of genomic variants. Of the ten remaining regions, six were present in the database of genome variants and were not investigated further. Two regions identified were not found in the database of genomic variants, but did not contain any genes, and were not identified in more than one patient sample.

In two patient samples we identified changes not completely represented in the database of genomic variants, each containing coding DNA sequences. Patient 1 carries a 344 kb pair duplication of chromosome 7p15.1 spanning bases 29,165,987–29,510,059 (Fig. 1A,C). The duplication contains the beta chimerin gene (*CHN2*; GenBank Accession: NM_004067). The proximal breakpoint of this duplication maps within intron 9 of the *CHN2* gene and as such could result in functional monosomy of the gene if the duplicated region is inverted or could result in a dominant negative allele if the duplicated region is in tandem. In patient 9 we identified a 324 kb pair duplication of chromosome 17p13.2 spanning bases 3,945,948–4,270,085 containing four genes (Fig. 1B,D). Three of the genes are wholly duplicated resulting in trisomy; cytochrome b5 domain containing 2 (*CB5D2*; GenBank Accession: NM_144611), ankyrin repeat and FYVE domain containing 1 (*ANKFY1*; GenBank Accession NM_016376), and ubiquitin-conjugation enzyme E2G 1 (*UBE2G1*; GenBank Accession: NM_003342). A fourth gene, zinc finger ZZ-type and EF hand domain containing protein 1 (*ZZEF1*; GenBank Accession NM_015113) spans the distal breakpoint of the duplication. Both of these novel regions were further studied using Taqman assays in order to validate the data and test parental DNA to determine segregation.

Oligo Array Analysis

244K Agilent oligo array assays were performed on seven patients from our cohort; the sample from Patient 11 was assayed with both the Affymetrix SNP and Agilent oligo arrays. Both genomic aberrations carried by Patient 11 identified by SNP analysis were confirmed by the oligo array. In addition, the oligo array identified 11 additional copy number variations in the sample from Patient 11 (see supporting information Table I which may be found in the online version of this article). All of the additional variants identified via oligo array in this sample were present in the database

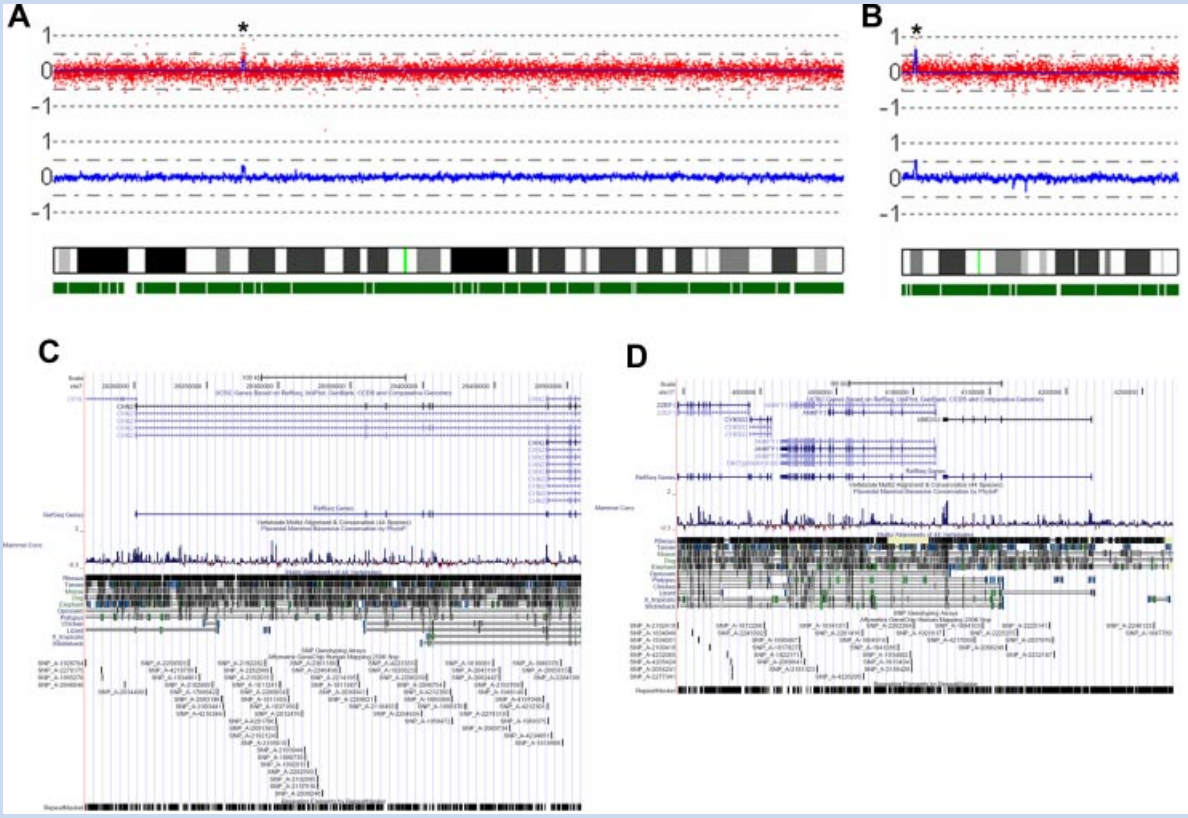

FIG. 1. Genomic duplications identified by SNP array. SNP data indicating the duplications carried by (A) Patient 1 on chromosome 7p15.1 and (B) Patient 9 on chromosome 17p13.2. The ideograms show the affected chromosome and banding patterns below the dosage of each SNP studied as indicated by red dots. The spike in the SNPs shows the duplications as indicated by an increase in the horizontal blue line and marked with an asterisk. The physical maps of the regions affected including the affected SNPs and genes are shown in (C) for Patient 1 and (D) for Patient 9 from the UCSC Genome Browser (http://www.genome.ucsc.edu). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

of genomic variants, and thus are not likely causative of the OEIS/EC phenotype.

Numerous CNVs were identified in the additional six patient samples (see supporting information Table I which may be found in the online version of this article). Patient 14 carries 23 changes, the highest number of identified changes within our cohort. In contrast, patient 11 carries 13 changes, the fewest we identified. We identified three CNVs each recurring in five different patient samples (see supporting information Table I which may be found in the online version of this article). These are well known copy number polymorphisms mapping to chromosomes 6p21.32 (HLA locus), 17q21.31 (also identified in our SNP array studies), and 22q11.23. In total our oligo array analysis identified an average of 19.2 changes per sample within our cohort. All of the changes identified via oligo array analysis were either present in the database of genomic variants or were deemed unlikely to be causative of the OEIS/EC phenotype based on the known or proposed function of the gene(s) therein and were not investigated further.

TaqMan Assays

TaqMan assays were performed in order to validate the novel DNA copy number changes identified by SNP chip analysis in Patients 1 and 9. The 344 kb pair duplication of chromosome 7p15.1 carried by Patient 1 was tested using a TaqMan assay mapping within intron 1 of the *CHN2* gene with a standard *RNase P* assay as a copy number control. The probe location within intron one is roughly located in the middle of the duplicated DNA segment. The *CHN2* TaqMan assay results confirmed the duplication identified in Patient 1 via SNP chip analysis. However, the assay also indicated that both parents are also heterozygous for this duplication and carry three copies of this locus (Fig. 2A).

A TaqMan assay located in the 3′ untranslated region of the *ANKFY1* gene was used to verify the duplication of chromosome 17p13.2 identified by SNP chip analysis in Patient 9. The *ANKYF1* TaqMan assay we performed is located roughly in the middle of the reported duplication. The assay when compared to the *RNase P* control probe indicated that the patient carries three copies of this locus (Fig. 2B). Our results also indicated that the father is heterozygous for this duplication, and the mother is actually homozygous for the duplication (has four copies). Thus, it is unlikely that this duplication is causally related to the OEIS/EC phenotype.

As an additional validation of our array CGH results, we also ran assay Hs05018555_cn mapping to chromosome 7p21.3. This assay detects a deletion carried by Patient 11 that was found by both the SNP and oligo array. The deletion was confirmed in Patient 11 and we found it was inherited from the patient's father (data not shown).

Candidate Gene Analysis

A candidate gene approach was undertaken to determine possible single gene mutations causing the OIES/EC phenotype. We performed a thorough search of the published literature to identify genes that are known to play a role in caudal dysgenesis and/or urogenital malformations in both human and mouse models. This search revealed 14 candidate genes for analysis (Table III). Direct DNA sequencing of all the exons and exon/intron boundaries of these 14 candidate genes did not reveal any disease-causing mutations.

We identified a total of 48 different DNA changes in our analysis. Thirty eight of these changes were single nucleotide polymorphisms (SNPs) that are present in the database of SNPs (dbSNP) and as such were deemed not disease causing and were not tested for parental segregation (Table IV). We also identified 10 DNA changes that were novel and not present in dbSNP (Table V). Six of these novel changes mapped to intronic DNA, did not uncover a cryptic splice site, and were not investigated further. Within the *IHH* gene we identified a single C–T transition within exon 3 in Patient 6, which changes an arginine to a leucine. However, the mother of this patient carries the same DNA change.

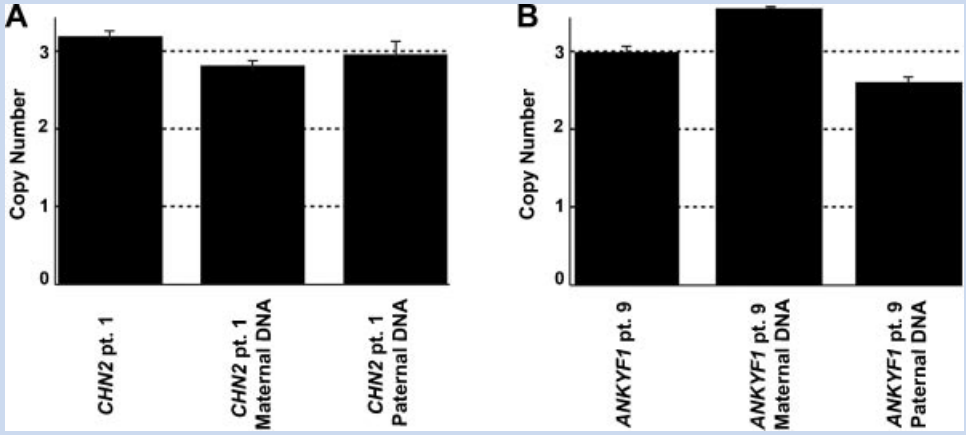

FIG. 2. Copy number analysis via TaqMan assay. (A) Assay Hs_04956436_cn mapping to the *CHN2* gene indicates a duplication carried by Patient 1 as well as both parents. (B) Assay Hs_0034272_cn mapping within the *ANKFY1* gene on chromosome 17 indicates that Patient 9 and her father carry three copies of this locus, while her mother carries four copies.

TABLE III. OEIS/EC Candidate Genes

Gene name	GenBank accession	Chr. Band	# exons	Sequencing rationale	Refs.
BMP4	NM_130851	14q22.2	4	Highly expressed in the tail bud, responsible for differentiation in developing kidney in mouse	Brenner-Anantharam et al. [2007]
CYP26A1	NM_000783	10q23.33	6	Knock-out mouse mutants die mid-gestation and show tail truncation, fused kidneys, blind ending hindgut, and abnormal urogenital sinus	Sakai et al. [2001]
DHH	NM_021044	12q13.12	3	*DHH* mutations in humans cause partial gonadal dysgenesis. *Dhh* null mice are infertile and have peripheral nerve abnormalities	Bitgood et al. [1996], Umehara et al. [2000]
EFNB2	NM_004093	13q33.3	5	*Efnb2* mutant mice display hypospadias, persitant cloaca, and anorectal malformations	Dravis et al. [2004]
FGF8	NM_033164	10q24.32	6	*Fgf8* null mice fail to gastrulate and die ~E10.5. Highly expressed in the tail bud	Crossley and Martin [1995], Sun et al. [1999]
IHH	NM_002181	2q35	3	*Ihh* mutant mice have malrotation, hypoplastic villi, and Hirschsprung-like phenotype in colon. *Ihh*/*Shh* double null mice have open gut	Ramalho-Santos et al. [2000], Zhang et al. [2001]
p63	NM_003722	3q28	14	*p63* null mice have bladder exstrophy. *p63* mutations in humans cause ectrodactyly, ectodermal dysplasia, and facial clefts and other malformation syndromes	Brunner et al. [2002], Cheng et al. [2006]
PCSK5	NM_006200	9q21.13	21	Mutation in mouse *Pcsk5* causes a VACTERL/caudal regression phenotype	Szumska et al. [2008]
SHH	NM_000193	7q36.3	3	Mutations of *Shh* in mouse cause duodenal stenosis, abnormal gut innervation, and imperforate anus. Important for formation of cloaca and hindgut	Haraguchi et al. [2007]; Ramalho-Santos et al. [2000]
T	NM_003181	6q27	8	Transcription factor required for proper mesoderm formation. Classic mouse mutation affecting tail length, sacral vertebrae, and the notochord	Herrmann et al. [1990]
TBX6	NM_004608	16p11.2	8	*Tbx6* null mouse embryos lack caudal somites and form ectopic neural tubes	Chapman and Papaioannou [1998]
WNT3	NM_030753	17q21.32	5	*Wnt3* null mice fail to form primitive streak and mesodermal tissue. Homozygous mutation of *WNT3* shown to cause tetra-amelia and urogenital defects in a single family	Liu et al. [1999], Niemann et al. [2004]
WNT3A	NM_033131	1q42.13	4	*Wnt3a* null mice have complete absence of tailbud, lack caudal somites, and have disrupted notochord; vestigial tail mouse mutant a hypomorphic allele of *Wnt3a*	Greco et al. [1996], Takada et al. [1994]
WNT9B	NM_003396	17q21.32	5	Regulates epithelial transition during urogenital organogenesis	Carroll et al. [2005]

Two changes were found in the 5′ untranslated region of the *WNT3* gene. In each case, the only parent available for analysis did not carry the change. Because only one parent was available we screened a panel of 100 DNA samples from apparently healthy individuals. The T–A transversion identified in Patient 14 was not found in 100 control samples, while the C–A transversion identified in patient 15 was identified in 1 of the 100 control DNA samples screened and thus is likely a rare polymorphism.

In the *FGF8* gene we identified an 18 bp duplication within exon 3 carried by Patient 9. Since the duplication does not shift the frame, the additional bases result in the duplication of 6 amino acids in the protein. Segregation analysis revealed that the mother of Patient 9 also carries the duplication in *FGF8*. One hundred control individuals of a similar ethnic background (Northern European Caucasian) were tested for this duplication, and none of the individuals carried the duplication. The six duplicated amino acids are not located in a conserved domain of the FGF8 protein.

A mutation in the 12S ribosomal RNA gene located on the mitochondrial chromosome (*MT-RNR1*; GenBank Accession NC_012920 region 648–1,601) has previously been reported in the proband from a family exhibiting maternally inherited pigment anomalies and aminoglycoside-induced deafness [Nye et al., 2000]. In addition the proband also presented with myelocystocele-cloacal exstrophy. The mitochondrial A1555G mutation was found in the proband (exhibiting deafness and EC) as well as all deaf members of the pedigree. We tested all patients in our cohort for the mitochondrial A1555G mutation. None of the patients in our OEIS/EC cohort carried this mitochondrial mutation.

TABLE IV. Known SNPs Identified During Candidate Gene Sequencing

Gene	dbSNP accession	DNA change	AA change	Location	No. pts. with het. change	No. pts. with hom. change
BMP4	rs2761880	A>G	N/A	Intron 3	0	13
	rs17563	T>C	V>A	Exon 2	5	6
CYP26A1	rs74151825	A>G	N/A	Intron 2	1	0
EFNB2	rs7995379	C>G	G>G	Exon 5	0	13
IHH	rs394452	G>A	T>T	Exon 3	5	7
	rs3731878	G>A	T>T	Exon 3	1	0
	rs3731881	T>C	P>P	Exon 3	7	6
P63	rs17514215	T>G	N/A	Intron 5	1	1
	rs33979049	C>T	L>L	Exon 6	1	0
	rs6790167	A>G	N/A	Intron 9	6	3
PCSK5	rs7020560	G>A	L>L	Exon 1	1	0
	rs7040769	T>C	C>C	Exon 1	3	0
	rs4745472	G>A	N/A	Intron 1	0	1
	rs2297344	C>A	N/A	Intron 7	4	2
	rs1416547	T>G	N/A	Intron 8	5	1
	rs2377429	T>C	N/A	Intron 8	6	0
	rs2297343	C>T	N/A	Intron 9	1	1
	rs41310061	G>A	T>T	Exon 9	1	0
	rs3824474	A>G	N/A	Intron 11	1	0
	rs2297342	G>A	S>S	Exon 12	2	2
	rs10124541	C>T	P>P	Exon 14	1	0
	rs2270570	A>G	N/A	Intron 16	5	1
	rs1537183	G>A	N/A	Intron 16	0	13
	rs7046850	T>C	N/A	Intron 17	1	0
	rs10869726	G>A	N/A	Intron 20	2	0
SHH	rs1233554	T>G	N/A	Intron 3	6	7
	rs1233555	G>A	N/A	Intron 2	9	4
T	rs1056048	C>T	S>S	Exon 3	2	2
	rs2305089	G>A	G>D	Exon 4	4	5
	rs9459599	G>A	N/A	Intron 5	2	2
	rs3127328	G>A	G>G	Exon 8	1	1
	rs35819705	G>A	A>A	Exon 8	0	2
TBX6	rs3809624	A>G	N/A	3′ UTR	2	0
WNT9B	rs4968281	T>C	T>M	Exon 2	3	8
	rs11654422	G>A	N/A	Intron 2	4	3
	rs11654424	G>A	N/A	Intron 2	4	3
	rs34072914	G>T	R>R	Exon 3	1	0
	rs754474	G>C	V>V	Exon 4	2	0

DISCUSSION

OEIS complex is a rare developmental field defect of unknown etiology. A significant genetic component is suspected based on the presence of familial cases and a higher concordance rate in monozygotic twins, yet no causative genes have been identified [Reutter et al., 2007b; Ludwig et al., 2009a]. The accumulating evidence for a strong genetic component as an underlying etiology for OEIS complex prompted us to perform a comprehensive genetic analysis of 13 individuals with OEIS complex. We sequenced 14 candidate genes and performed high resolution genomic arrays using either a 250K SNP array or a 244K oligonucleotide array. No causative mutations or genomic aberrations were identified in our patient cohort.

OEIS complex has been reported in association with Trisomy 21, Trisomy 18, Triple X syndrome, Turner mosaicism, and 1p36 deletion [Ludwig et al., 2009a; El-Hattab et al., 2010]. However, given the relatively high prevalence of these chromosomal disorders in the general population, it is unlikely that OEIS complex is causally associated with these chromosomal aberrations. Two patients with cloacal exstrophy and chromosomal deletions have been reported: one patient carrying an unbalanced translocation between chromosome 9 and the Y chromosome, resulting in monosomy for chromosome 9q34.1-qter, and one patient with del(3)(q12.2q13.2) [Thauvin-Robinet et al., 2004; Kosaki et al., 2005]. One additional patient with OEIS complex and hypomelanosis of Ito has been reported [Leonard and Tomkins, 2002]. This patient was mosaic for a cell line with t(1;6) (p32;q13) in fibroblasts.

TABLE V. Novel SNPs Identified During Candidate Gene Sequencing

Gene	Patient	DNA change	Amino acid change	Location	Nomenclature[a]
CYP26A1	13	G>C	N/A	Intron 2	IVS2+86G>C
	15	G>C	N/A	Intron 2	IVS2−99G>C
FGF8	9	18 bp duplication	6 duplicated	Exon 3	c.104_121dupAGCTCGCTTCCCTGTTCC
IHH	15	C>T	N/A	Intron 1	IVS1+49C>T
	05	G>A	R>L	Exon 3	c.1169G>A
PCSK5	05	T>A	N/A	Intron 16	IVS16+58T>A
SHH	14	C>T	N/A	Intron 3	IVS3−70C>T
WNT3	14	T>A	N/A	5′ UTR	c.−73T>A
	15	C>A	N/A	5′ UTR	c.−86C>A
WNT9B	9	T>C	N/A	Intron 2	IVS2+86T>C

[a]Nomenclature based on following accession numbers: *CYP26A1*:NM_000783, *FGF8*:NM_033163, *IHH*:NM_002181, *PCKS5*:NM_006200, *WNT3*:NM_030753, *WNT9B*:NM_003396.

A causal relationship between this translocation and OEIS complex is unclear. Chromosomal aberrations have also been reported in patients with BE and epispadias [Ludwig et al., 2009a]. However, no recurrent chromosomal aberrations have been identified either for OEIS complex or BE.

Several other studies examining candidate genes in patients with OEIS complex or BEEC have been performed. In general, these studies have examined one candidate gene in a small number of individuals. The homeobox gene *HLXB9*, which is mutated in Currarino syndrome (OMIM #176450) [Ross et al., 1998], was sequenced in bladder DNA of five patients with OEIS/EC and leukocyte DNA from five patients with bladder exstrophy, and no mutations were identified [Boyadjiev et al., 2004]. Because of their respective genomic localizations near a reported translocation in a patient with OEIS complex, the *NR5A1* (Steroidogenic factor-1) gene on 9q33.3 was analyzed in 1 patient with OEIS complex [Thauvin-Robinet et al., 2004], and the *SET* gene (Suppressor of variegation, Enhancer of Zeste and Trithorax) on 9q34.11 was analyzed in 33 patients with BEEC [Reutter et al., 2006]. No causative mutations were identified in either gene. Because of its localization at 9q13, which has been implicated in two independent translocations in BE, the *CNTNAP3* gene was analyzed in several patients with BE, but no association was found [Boyadjiev et al., 2005]. The *FGF10* gene was analyzed in 10 patients with OEIS because of its known role in urethral plate fusion based on mouse knockout models [Kruger et al., 2008]. Similarly, the *TRP63* gene was analyzed based on the bladder exstrophy phenotype of the ΔNp63 knockout mouse [Cheng et al., 2006; Ching et al., 2007]. Again, no causative mutations were identified in either gene. In one patient with OEIS complex and aminoglycoside-induced sensorineural hearing loss, a mutation in the mitochondrial 12S RNA gene was identified [Nye et al., 2000]. However, the mutation in this family segregated with the hearing loss phenotype. Although other family members carrying the 12S RNA mutation had minor urogenital anomalies, a causative relationship between the 12S RNA gene and OEIS complex could not be established. Furthermore, this mutation was not identified in any of the patients in our cohort.

The candidate genes in this study were chosen based on several different criteria. (1) Mouse knockout models with caudal or urogenital phenotypes, (2) a known role for the candidate gene in caudal and/or urogenital development in humans, or (3) embryonic expression patterns which suggest a potential role in caudal and/or urogenital development. We did not identify any point mutations that were unequivocally causative of the OEIS/EC phenotype. We did identify several known SNPs in our study, as well as a number of novel DNA changes. With the exception of variants in the 5′ untranslated region of the *WNT3* gene in two patients, all of the other novel DNA changes were also carried by at least one unaffected parent. The novel variants identified in the *WNT3* gene were not present in an unaffected parent, although only one parent was available for analysis. Although mutation of *WNT3* has been associated with urogenital malformations and tetraamelia in one consanguineous family [Niemann et al., 2004], this recessive nonsense mutation likely led to loss of function of the gene, and the heterozygous carrier parents were normal. Intriguingly, the *FGF8* duplication variant was not found in a control population of 100 individuals (200 chromosomes). Although singularly none of these changes are likely sufficient to cause OEIS/EC, we cannot rule out that they are associated with the disease phenotype. However, the rarity of this disorder limits our ability to obtain a sufficiently large patient cohort to perform the necessary statistical analysis to adequately test this hypothesis.

Only limited whole genome studies in patients with OEIS complex have been reported. One patient carrying a deletion of 3q12.2q13.2 was identified using bacterial artificial chromosome aCGH [Kosaki et al., 2005]. A copy number variant (CNV) was identified in a consanguineous family with three affected members with BE; however, this was a well known CNV on 1p21.1 encompassing the pancreatic and salivary amylase genes; thus it is unlikely related to the BE phenotype [Reutter et al., 2007a]. More recently, a genome-wide association study was performed in two BE families: one nonconsanguineous family with two affected family members and one consanguineous family with two affected family members [Ludwig et al., 2009b]. Although several genomic loci were identified with LOD scores of >1.5, a causative role for any of these regions remains to be established. Furthermore, these studies are based on an autosomal recessive mode of inheritance for BEEC, which has not definitively been established in the literature.

We utilized two different aCGH platforms in our study: a 250K SNP array and a 244K oligonucleotide array. We observed greater

sensitivity with the oligonucleotide array. In the one sample that was analyzed on both platforms, the two CNVs that were identified by the SNP array were also identified by the oligo array, as well as 11 additional CNVs. However, higher density SNP arrays are now available that may improve the sensitivity of the SNP arrays. While we identified numerous CNVs in our patient cohort, the majority were known CNVs that are well represented in the database of genome variants. In addition, the only recurrent CNVs in our patient cohort are well known to be widely polymorphic. The CNVs that we identified that were not represented in the database of genome variants were both validated by TaqMan assay and were found to be present in at least one unaffected parent. Therefore, individually, none of the CNVs that we identified are likely to be causally associated with the OEIS complex phenotype. However, we cannot rule out the possibility that one or more of these CNVs may be a genetic risk factor for development of the OEIS complex phenotype in combination with other environmental or genetic risk factors [Klopocki et al., 2007]. Further, it is also possible that one of the CNVs could have unmasked a recessive mutation at one of these loci. Sequencing of the candidate genes contained within these CNVs in the other affected individuals could help to clarify this possibility.

Based on our current knowledge of OEIS complex, the majority of individuals represent sporadic cases, and the recurrence risk for future pregnancies is thought to be low. Although other modes of inheritance cannot be completely excluded, this is most consistent with a de novo mutation in a gene that affects development of caudal and urogenital structures. Because so few affected individuals reproduce, it is difficult to know whether these affected individuals would pass along the complete OEIS complex phenotype to their offspring. For the few familial cases, the inheritance pattern has been most consistent with autosomal dominant inheritance with reduced penetrance and/or variable expressivity, influenced either by other genetic or epigenetic factors [Keppler-Noreuil, 2001; Boyadjiev et al., 2004]. Recently, it has been proposed that the Disorganization gene (*Ds*) in mice may represent a model for OEIS complex including limb malformations in humans [Robin et al., 2007]. *Ds* mice have variable phenotypes that can resemble OEIS complex, and the penetrance is known to be low. However, since the *Ds* gene has not yet been identified, it is impossible to prove or disprove this interesting theory.

At this time, the pathogenesis of OEIS complex remains elusive. The lack of a single genetic etiology precludes the creation of an animal model, and the lack of an animal model that completely recapitulates the phenotype limits the ability to study developmental pathways and isolate candidate genes. It is most likely that OEIS complex is a developmental field defect of the cells comprising the caudal eminence, and perturbation of this region at specific time points during development, either by genetic or environmental factors, leads to the OEIS complex phenotype.

While no causative genetic explanation was found in this study, it is still likely that there are as of yet unidentified genes that strongly contribute to the OEIS complex phenotype, and additional research is needed. Although a large number of candidate genes were analyzed in this study, we cannot rule out that a mutation in one of these genes could cause an OEIS complex phenotype in rare patients, or that some of the rare variants that were found are associated with the disease phenotype and have reduced penetrance in unaffected parents. While we did not identify any unique recurrent CNV that might contain a candidate gene for OEIS complex, we also cannot exclude the possibility that rare CNVs might cause OEIS complex. However, it is unlikely that OEIS complex is caused by a recurrent genomic CNV.

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