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Variants in *ALX4* and their association with genitourinary defects

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Abstract

Background: Genitourinary anomalies occur in approximately 1% of humans, but in most cases, the cause is unknown. Aristaless-like homeobox 4 (*ALX4*) is an important homeodomain transcription factor. *ALX4* mutations in humans and mouse have been associated with craniofacial defects and genitourinary anomalies such as cryptorchidism and epispadias.

Objectives: To investigate the presence and the functional impact of *ALX4* variants in patients with genitourinary defects.

Materials and methods: Two separate patient cohorts were analyzed. One includes clinical exome-sequencing (ES) data from 7500 individuals. The other includes 52 *ALX4* Sanger-sequenced individuals with bladder exstrophy-epispadias complex (BEEC). Dual luciferase assays were conducted to investigate the functional transcriptional impact of *ALX4* variants in HeLa cells and HEK293 cells.

Results: A total of 41 distinct *ALX4* heterozygous missense variants were identified in the ES cohort with 15 variants present as recurrent in multiple patients. p.G369E and p.L373F were the only two present in individuals with genitourinary defects. A p.L373F heterozygous variant was also identified in one of the 52 individuals in the BEEC cohort. p.L373F and p.G369E were tested in vitro as both are considered damaging by MutationTaster, although only p.G369E was considered damaging by PolyPhen-2. p.L373F did not alter transcriptional activity in HeLa and HEK293 cells. p.G369E caused a significant 3.4- and 1.8-fold decrease in transcriptional activities relative to wild-type *ALX4* in HEK293 and HeLa cells, respectively.

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AUTHOR CONTRIBUTIONS

CHC and JZ carried out mutagenesis and luciferase assays, participated in the analysis, and drafted the manuscript. JCB and NW carried out cloning, mutagenesis, and luciferase assays, participated in the analysis, and drafted the manuscript. JAR carried out the genetic studies, participated in the analysis, and drafted the manuscript. AS consented patients, participated in the analysis, and drafted the manuscript. CJJ designed the project, consented patients, participated in the analysis, and drafted the manuscript. All authors read and approved the final manuscript.

Discussion and conclusions: Our study supports the idea that transcription factors like *ALX4* could influence the normal development of the GU tract in humans as demonstrated in mouse models as *ALX4* variant p.G369E (predicted pathogenic by multiple databases) affects *ALX4* function in vitro. Variant p.L373F (predicted pathogenic by only MutationTaster) did not affect *ALX4* function in vitro. Exon-sequence information and mouse genetics provide important insights into the complex mechanisms driving genitourinary defects allowing the association of transcriptional defects with congenital disorders.

Keywords

ALX4; bladder exstrophy-epispadias complex (BEEC); epispadias; genitourinary; micropenis; single nucleotide variants (SNV)

1 | INTRODUCTION

At least 1% of human fetuses will have congenital anomalies affecting the genitourinary (GU) system.[1] The frequency of reports of GU malformations has increased in recent decades.[2] Advances have been made regarding the discovery of the genetic causes of GU defects, especially in upper malformations in which mutations in over 40 single genes cause kidney disease in 5%−20% of patients.[3,4] Unfortunately, less is known about genetic causes of lower GU tract anomalies. Birth defects of the lower GU system affect the bladder, urethra, or genitalia and range from rare anomalies, such as bladder exstrophy-epispadias complex (BEEC), and prune belly syndrome to common ones such as hypospadias and cryptorchidism. A subset of these defects can be linked to environmental and hormonal signal perturbation.[5–7] A small subset of genes has been established as important for the development of the lower GU tract.[1,8–12] Although the common GU defects such as hypospadias and cryptorchidism are not life-threatening, they must be repaired surgically and can carry potential long-term sequelae such as infertility, cancer, and, possibly, because of underlying pleiotropic causes, an increased risk for neurodevelopmental disorders, including autism. Consequently, their etiology is an important subject of investigation.[13–16]

One of the most complex birth defects involving the lower GU tract is BEEC. BEEC ranges from the more severe phenotypes of cloacal exstrophy (CE) and classic bladder exstrophy (CBE) to the less severe phenotype of epispadias.[17] Historically, BEEC malformations were fatal disorders, but advances in medical care have improved patient survival and quality of life.[18] Transgenic mouse models serve as a useful tool for understanding the molecular mechanisms underlying complex diseases such as BEEC. Unfortunately, because of the severity of BEEC phenotypes, most mouse models are expected to be embryonic or perinatal lethal, and the association between gene defect and phenotype is difficult to establish. An example of such a mouse model of BEEC is the *Tp63*-null mouse that dies several hours after birth because of multiple anomalies that include absence of limb and craniofacial abnormalities.[19] Several years after the report by Mills et al, it was reported that *Tp63*-null mice had features that mimic human BEEC.[20] Epispadias has been described in mice homozygous for a targeted mutation of aristaless-like homeobox 4 (*Alx4*).[21] Most *Alx4*-null mice die at birth as the result of gastroschisis.[22] Genetic and physical mapping identified *Alx4* as the gene responsible for the *Strong's luxoid* (*Lst*) phenotype

described in mutants mice.[23] There are several *Lst* mouse lines that arose spontaneously or during mutagenesis screenings.[24,25] The penetrance and the expressivity of the different *Lst* phenotypes are sensitive to genetic background, with homozygous mutant mice having a range of phenotypes that includes polydactyly of all four limbs, craniofacial defects, dorsal alopecia, weakness of the ventral body wall, open eyelids at birth, and, in males, anomalies of the phallus and cryptorchidism.[26–29]

Mutations in and/or deletions of *ALX4* have been associated with several developmental disorders, such as autosomal dominant parietal foramina,[30–34] autosomal recessive frontonasal dysplasia,[35–37] craniosynostosis,[38] and Potocki-Shaffer syndrome, which can include GU defects.[30,39,40] As the mouse *Alx4* phenotype involves GU defects ranging from common cryptorchidism to rare epispadias, and because these defects intersect with our interest in understanding the genetic basis of GU defects, variants in *ALX4* were investigated in a cohort of individuals undergoing clinical exome sequencing (ES) as well as a different cohort of 52 children who presented with BEEC. As ALX4 is an important transcription factor, the transcriptional impact of two *ALX4* variants was investigated in vitro using site-directed mutagenesis and luciferase reporter assays.

2 | METHODS AND MATERIALS

2.1 | Selection of study patients

This study was approved by and under the oversight of the Institutional Review Board of Baylor College of Medicine (BCM), Houston, TX. Two different patient populations were analyzed. One group included 52 patients with BEEC (11 with epispadias, 30 with CBE, and 11 with CE). Subject data and specimens were obtained following informed consent by the subjects at Texas Children's Hospital (TCH). Approved informed consents were obtained from the appropriate parents or legal guardians. A sample of blood was obtained at the time of consent when the patients underwent surgery. Saliva samples were collected from the parents. DNA was extracted from all samples using the Qiagen Puregene DNA extraction kit according to the manufacturer's protocol.[41] Data from the other cohort included ~ 7500 individuals undergoing clinical ES at the Baylor Genetics (BG) Laboratory[42] that were examined for all called variants in *ALX4*. The ES protocol, including library construction, exome capture by VCRome version 2.1, HiSeq next-generation sequencing, and data analysis, was developed by the Human Genome Sequencing Center at Baylor College of Medicine and adapted for the clinical test of ES.[43,44] Individuals in this group have diverse clinical manifestations, most often including nervous system dysfunction such as developmental delay.[42]

2.2 | SNV detection

From the cohort of 52 patients with BEEC, the 4 exons of *ALX4* were PCR amplified and Sanger-sequenced. No other genetic information was available from these patients. For PCR reactions, 50 ng of gDNA was amplified using Phusion High-Fidelity PCR Master Mix with GC Buffer (NEB). After PCR, the product was purified using the ExoSAP-It kit (USB Scientific). Purified products were sequenced using Sanger sequencing combined with ABI 3730*xl* DNA analyzers for capillary electrophoresis and fluorescent dye terminator detection

(GENEWIZ). Data were analyzed using Mutation Surveyor (SoftGenetics). Bioinformatics assessment to determine the potential pathogenicity of the mutation was performed using PolyPhen-2,[45] SIFT-Provean,[46] and MutationTaster.[47] Primer sequences are indicated as follows: (a) Exon1F: cagagaaagaagaggggaga, (b) Exon1R: cgggaaaaacaaactccaag, (c) Exon2F: ggtgaagaggctcagattgg, (d) Exon2R: gtcgctctagttgggacagg, (e) Exon3F: gaacagtttgcactgcctga, (f) Exon3R: ggcctcccacactctggtat, (g) Exon4F: aacactcaagaggggcttca, (h) Exon4R: caggccaggttcctaagagg.

2.3 | Site-directed mutagenesis

Human *ALX4* plasmid ID HsCD00294887 was obtained from Harvard Medical School. The *ALX4* open reading frame was subcloned into the expression vector pcDNA3.1 (Invitrogen) to generate the pcDNA3.1-*ALX4* expression plasmid. The p.L373F and p.G369E single nucleotide variants in the pcDNA3.1-*ALX4* expression plasmid were generated using the QuikChange Lighting Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer's protocol. Mutations were validated through Sanger sequencing (GENEWIZ).

2.4 | Luciferase assay

pGL3-P3-Luc was a generous gift from Dr Paul A. Hamel.[48] Each variant of *ALX4* was co-transfected with pGL3-P3-Luc and Renilla vectors (pRL-TK Promega) into human HeLa and HEK293 cells. A concentration of 500 ng of *ALX4* construct DNA was used for each transfection. HeLa and HEK293 cells were seeded at 300000 cells/ well in 12-well plates and incubated overnight. Transfection mixtures were prepared in Opti-MEM (Thermo Fisher Scientific) using Lipofectamine 3000 (Thermo Fisher Scientific) per manufacturer's instructions, and transfection was conducted per manufacturer's protocols. As a positive control, we used pGL3-control plasmids (Promega), which provided high readings of luciferase activity, and all transfections were done in conjunction with a Renilla expression plasmid as an internal control. Cell lysates were collected 24 h post-transfection using 1X Passive Lysis Buffer, which was provided as a part of the Promega Dual-Glo Luciferase Assay Kit (Promega). Cell lysates were centrifuged, and the supernatants were utilized for reading. Luciferase activities were normalized to Renilla activities. The luciferase assays were conducted independently in triplicate and compiled. Promoter-less plasmid (pGL3-Basic) and pRL-TK Renilla luciferase activities were not affected by any of the *ALX4* constructs (data not shown). The luciferase readings for mutated *ALX4* were normalized for transfection efficiency, compared with the wild-type (WT) *ALX4*, and presented as normalized luciferase units. Each biological replicate has two technical replicates. These data were used to determine the statistical significance for each construct of the luciferase assays using GraphPad Prism ANOVA. The results were shown as the mean ± standard error of the mean (SEM).

3 | RESULTS

3.1 | *ALX4* variants identified in patients with genitourinary (GU) tract defects

We surveyed variants in *ALX4* among 7,500 individuals undergoing clinical ES.[42] Most individuals in this group have indications of nervous system dysfunction, with only 350 (4.7%) having an indication of lower GU tract defect [cryptorchidism or undescended testis

(207 cases), hypospadias (106 cases), micropenis (32 cases), genitalia defects (38 cases), sex reversal (two cases), urogenital defects (one case), epispadias (zero case), exstrophy (three cases), and cloacal abnormalities (six cases)]. Forty-one distinct missense single nucleotide variants (SNV) in *ALX4* were identified in 228 individuals (3.0%) Figure 1. Twenty-six variants were identified in only one individual with 46.2% of these predicted to be pathogenic by PolyPhen-2. Seven of these variants were novel as they had not been reported in any database,however, as none of the individuals carrying these variants had a GU phenotype, they were not studied in vitro. The remaining 15 SNV seen more than once are shown in Table 1. With the exception of the two probands carrying the predicted benign p.Q111P variant, a subset of the probands carrying any of these 15 variants had part of their phenotypes explained by a variant or CNV in genes other than *ALX4*. Three variants were observed in individuals with genital anomalies (p.R63Q, p.G369E and p.L373F). p.R63Q was detected in six individuals, with only one having an unclear diagnosis of genital anomalies,however, it was not possible to determine the exact anomalies in this proband. Therefore, this variant was not investigated further. The other two SNVs (p.G369E and p.L373F) were present in at least 10 individuals, and both of them were considered damaging according to MutationTaster.[47] However, only p.G369E was considered damaging when PolyPhen-2 was used. All individuals with these SNVs had phenotype information available that is indicated in Tables 2 and 3. Although this study focused on SNVs, we noticed a deletion of 12 nucleotides (p.P105_Q109del) in 113 individuals, 10 of whom had a GU phenotype ranging from common to rare including ambiguous genitalia, BEEC, and urogenital sinus. This deletion was not further investigated because it is commonly reported, and GU abnormalities were not over-represented among carriers.

As *Alx4*-mutant mice had epispadias, the four exons of *ALX4* were sequenced in patients with BEEC defects: 11 with epispadias, 30 with CBE, and 11 with CE. Sanger sequencing identified two synonymous (p.L293L and p.A243A) and a de novo missense heterozygous variant p.L373F Figure 2A–C. The frequency of p.L373F is 223 of 281,712 alleles (MAF = 0.00079, including one homozygote) in a presumed healthy general population (gnomAD database) with a higher incidence in the African population (0.00820) followed by Latino population (0.00034). The frequency of p.L373F SNV in gnomAD in males (n = 76 531) compared with that in females (n = 64 325) is significantly lower (92 vs 131). In our ES population, we observed a higher number of males carrying this variant (nine of 14). This variant was also identified in three other databases with similar incidences Table 2. Of the 7500 individuals undergoing clinical ES, 14 (0.1% allele frequency) of them carried p.L373F with at least 5 of them being of African descent. The 14 individuals presented a diverse range of phenotypes, with 9 of them having phenotypes observed in *Alx4* mutant mice and two of them having genitalia defects Table 2. Chi-square testing indicates that the presence of this variant was not significantly different in our ES cohort from that in the gnomAD database ($P = .6506$). Of the two boys with lower genital defects, one of them has cryptorchidism and the other has sex reversal associated with androgen deficiency. The percentage of boys with cryptorchidism carrying this variant in the ES database was small (1/207, 0.5%). The frequency of cryptorchidism among carriers of p.L373F was not enriched, as compared with the ES cohort as a whole ($P = .8545$).

A heterozygous p.G369E variant was identified in 10 (five males and five females) of the 7500 individuals undergoing ES (0.07% allele frequency), with one of them having GU defects Table 3. This variant was not seen in dbSNP or the 1000 Genomes database. In gnomAD, the allele count for p.G369E was 155 of 281 568 total alleles (MAF = 0.00055, no homozygotes), mostly seen in female 106 (68.4%). The incidence of this heterozygous variant in females (n = 64 271) in the general population is significantly different ($P < .0001$) from that in males (n = 76 513)-. p.G369E is predicted to be damaging by PolyPhen-2 (score of 0.960 in HumVar and 0.998 in HumDiv), SIFT, and MutationTaster. Sanger sequencing of the fetus with micropenis in the ES cohort indicates that this variant is maternally inherited Figure 2D–F. The incidence of micropenis in the general population is 1.5:10.000,[49] and one of the 32 boys (3.1%) with micropenis in the ES database carries this variant. Chi-square testing based on the presumed healthy individuals in the gnomAD database indicated that p.G369E was not significantly different in our ES cohort from that in the control databases ($P = .6807$). However, the frequency of micropenis among carriers of p.G369E was greater than the frequency in the ES cohort as a whole ($P = .0291$).

3.1.1 | Variant p.G369E has loss of transcriptional activity—

ALX4 is a homeodomain protein that can bind with palindromic repeats of a consensus monomer binding sequence (5'TAAT3') separated by a variable number of nucleotides. ALX4's highest affinity is toward palindromic DNA sequences, composed of two inverted TAAT half sites and separated by a 3-basepair nucleotide spacer, TAATnnnATTA, called the P3 binding element.[38,48,50] ALX4-P3 reporter assays have been used to test the pathogenicity of different *ALX4* variants (p.V7F, p.K211E, and p.P306L).[38] To study the functional consequences of p.L373F and p.G369E, these mutations were introduced in a mammalian expression vector that contained human *ALX4* cDNA under the control of a CMV promoter (pcDNA3.1-*ALX4*). Each of these vectors was co-transfected with a reporter vector (pGL3-P3-Luc), which contains a P3 responsive element (*ALX4*-binding element) controlling the expression of the firefly luciferase reporter gene. The transcriptional activity of p.L373F and p.G369E was measured and assessed in two different cell types previously used to test *ALX4* expression and function.[50,51] p.G369E exhibited a 3.4- ($P < .0001$) and 1.8-fold ($P < .01$) decrease in transcriptional activities relative to controls in HEK293 and HeLa cells, respectively Figure 3. The p.L373F variant did not alter transcriptional efficiency in either cell type ($P > .05$) when compared with control.

4 | DISCUSSION

ALX4 is an important developmental homeobox gene composed of 4 exons with a highly conserved carboxy-terminal regionFigure 1D.[31] *ALX4* variants inside and outside the homeobox region are associated with disease. Variants causing parietal foramina are present in both inside (p.Q246X, p.R216Q, p.R218Q, p.R272P, p.S207X, p.R216G) and outside the homeobox region (p.Q140X).[31,32,34,38] p.V7F and p.K211E associated with craniosynostosis and outside the homeobox region have been shown in vitro to have a gain-of-function effect that could be because of loss of interaction of *ALX4* with its upstream repressor TWIST1.[38] In silico prediction tools have high sensitivity but low specificity.[52–54] A study using PolyPhen2 in 2,314 *TP53* missense variants indicated that from the variants

predicted to be deleterious, only 58% were true-positive, but of the variants predicted to be benign, 96% were true-negative.[52] As the incidence of true-negative variants is very high when PolyPhen2 is used, the in vitro function of only one predicted benign variant (p.L373F) was tested. p.L373F was selected because despite being predicted as benign by PolyPhen2, it was predicted to be damaging by MutationTaster, and it was identified de novo in a boy with epispadias. As predicted by PolyPhen2, p.L373F did not affect *ALX4* DNA-binding and transcriptional activity of ALX4. However, p.L373F may still affect additional ALX4 functions such as interactions with other proteins, and only future in vivo experiments will be able to prove the pathogenicity of this variant.

The predicted pathogenic variant, p.P306L (which was seen in multiple individuals in our ES cohort, but in none noted to have GU defects), was tested in vitro by a different group of investigators and showed no effect.[38] We tested the predicted pathogenic variant, p.G369E, and found a decrease in luciferase activity, suggesting that this variant could affect the transcriptional activity of ALX4. The only individual carrying *ALX4* p.G369E with a genital defect (micropenis) was a fetus. It is unknown if this proband had additional penile or genital defects,however, anomalies observed in *Alx4*-deficient mice were also present (skeletal abnormalities, mild platyspondyly, fifth finger clinodactyly, bilateral club feet, mild brachycephaly, hypotelorism, and cleft lip/palate). In addition, the fetus exhibited kidney abnormalities. Three additional individuals carrying p.G369E had kidney and ureter abnormalities Table 3. This variant is rare with a low incidence in gnomAD (MAF = 0.00055), especially in females (68.4%). The fetus with micropenis inherited the variant p.G369E from his mother. Defects of genitalia are common in males (1:200 for hypospadias), but uncommon in females (1:448 000 for epispadias in females),[55] suggesting that p.G369E could be associated with defects of genitalia, but as such defects are not common in females, the defect is less frequently reported.

ALX4 p.V7F and p.K211E were inherited from a presumably unaffected parent.[38] Some *ALX4* variants considered disease-associated (p.R216Q, p.R218Q, p.S207X, p.V7F, and p.K211E) are present in presumed healthy individuals in the gnomAD, Go-ESP, TOPMed, and/or 1000-genomes databases, suggesting an incomplete penetrance of the phenotypes caused by *ALX4* variants or perhaps the existence of undiagnosed patients, probably because of mild symptoms. The pLI score (probability of loss-of-function intolerance) for *ALX4* is not indicative of intolerance to loss of function (0.36), although the observed/expected (o/e) ratio is low (0.23, 90% CI 0.11–0.52), supporting some selection against loss-of-function variants. Some of these variants, such as heterozygous p.R272P that disrupts DNA binding, are autosomal dominant.[31] Others, like p.Q225E, are considered autosomal recessive.[36] Novel homozygous *ALX4*p. Q98Sfs*83 was present in two unrelated boys with frontonasal dysplasia type-2 and monorchism. One of the boys inherited the variant from his heterozygous father who had a unilateral atrophic testis indicating possible pathogenicity of the variant in a heterozygous state.[37] The mode of inheritance of *ALX4* variant seems to be complex. In some cases, is it autosomal recessive, in others autosomal dominant, and in others could be polygenic.

The variant p.G691S in the *RET* gene is a common polymorphism (allele frequency of 20% in dbSNP database) associated with VUR. However, in conjunction with variants p.R982C

in *RET* or p.R93W in *GDNF,* it is associated with severe renal defects demonstrating that *RET* p.G691S alone has a mild or moderate effect but modulates the penetrance of other variants to cause a severe defect.[56,57] Similar to the *RET* variant, *ALX4* missense variants could act as a modifier. In the heterozygous state, sufficient ALX4 functionality could remain so that carriers will not always manifest an abnormal GU phenotype, but when combined with other variants and/or genetic backgrounds (multifactorial inheritance), result in occurrence of an abnormal GU phenotype. Therefore, GU birth defects could be multifactorial, and the observed *ALX4* variants are low-penetrance mutations that in a heterozygous state predispose individuals to, but do not cause, GU malformations by themselves. The incomplete penetrance for *ALX4*-associated phenotypes is supported by animal data.[22] Even though 98% of *Alx4* null mice lack ventral abdominal wall musculature and die, 2% of *Alx4* null mice survive because the ventral body wall defect is small enough to heal spontaneously.[22] This suggests variable expressivity of the phenotype and could be the reason for the diversity of phenotypes observed in our patients. Many of the described copy number variants and mutations related to GU defects and BEEC are rare and in some cases inherited from a supposedly healthy parent.[11,57,58] Similar low-penetrance mutations have been observed in other candidate genes for BEEC, such as *TP63*.[59] The increasing use of ES as well as copy number variants information continues to provide insights into the disorders of transcriptional regulation. Such insights have contributed significantly in our understanding of the etiologies of malformation syndromes caused by defects in transcription factor. In conclusion, our work supports the idea that transcription factors like *ALX4* could influence the normal development of the GU tract in humans as demonstrated in mouse models.

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CONFLICT OF INTERESTS

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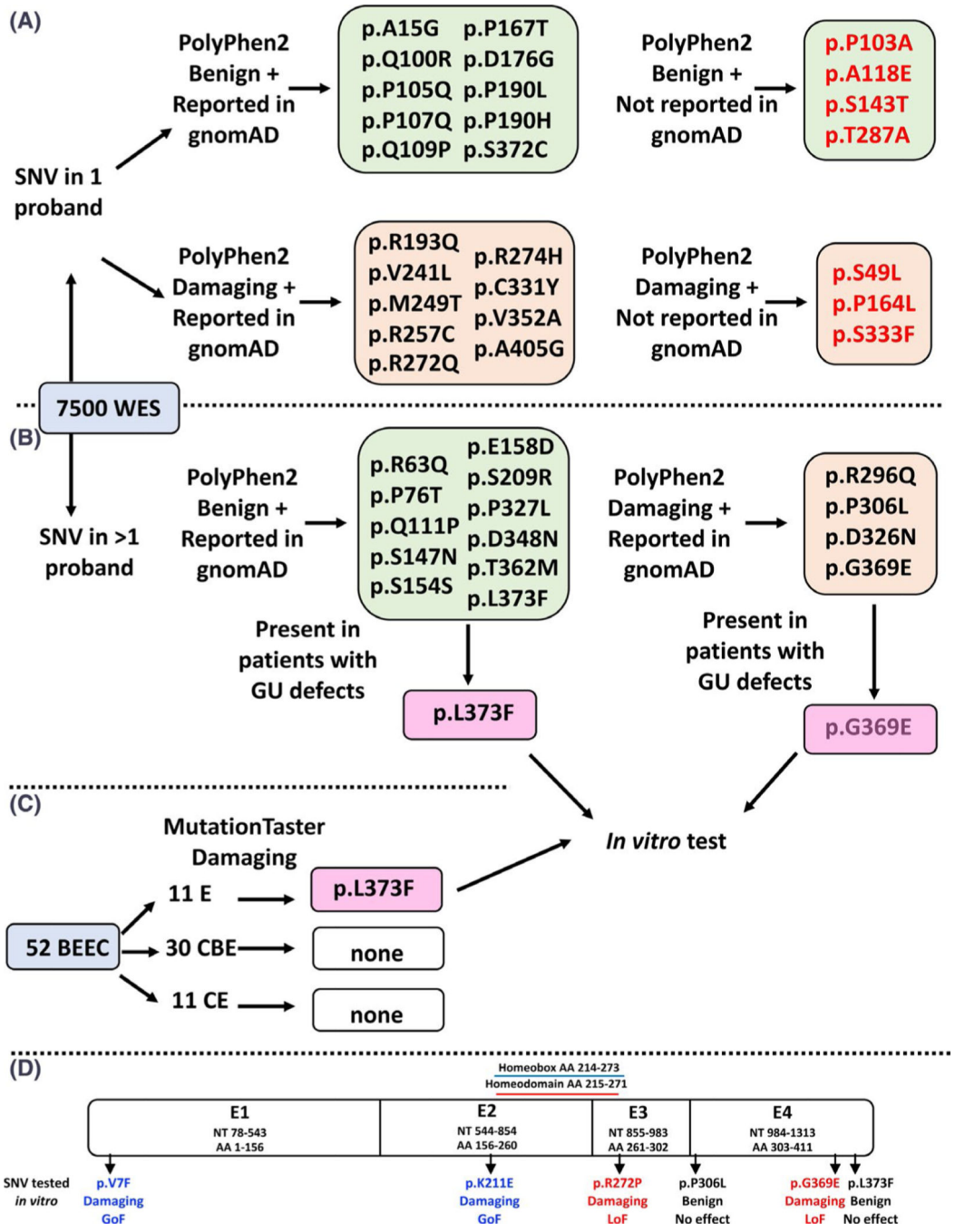

FIGURE 1.

Workflow of analysis of missense single nucleotide variants (SNV) identified in *ALX4* in a database of 7500 ES clinical cases and a cohort of 52 probands with BEEC. Variants in black had been reported in gnomAD, and the ones in red are not present in gnomAD. Variants from ES were classified according to presence in only one proband (A) or more than one proband (B). In addition, variants were classified on the basis of PolyPhen2 prediction as benign (green boxes) or damaging (orange boxes). Variants present in patients with specified lower GU defects (pink boxes) were selected for in vitro testing. (C) Sanger

sequencing of individuals with BEEC. (D) SNV in the 4 *ALX4* exons tested in vitro by our group as well as Yagnik et al[38] Gain of function (GoF) and loss of function (LoF) are indicated

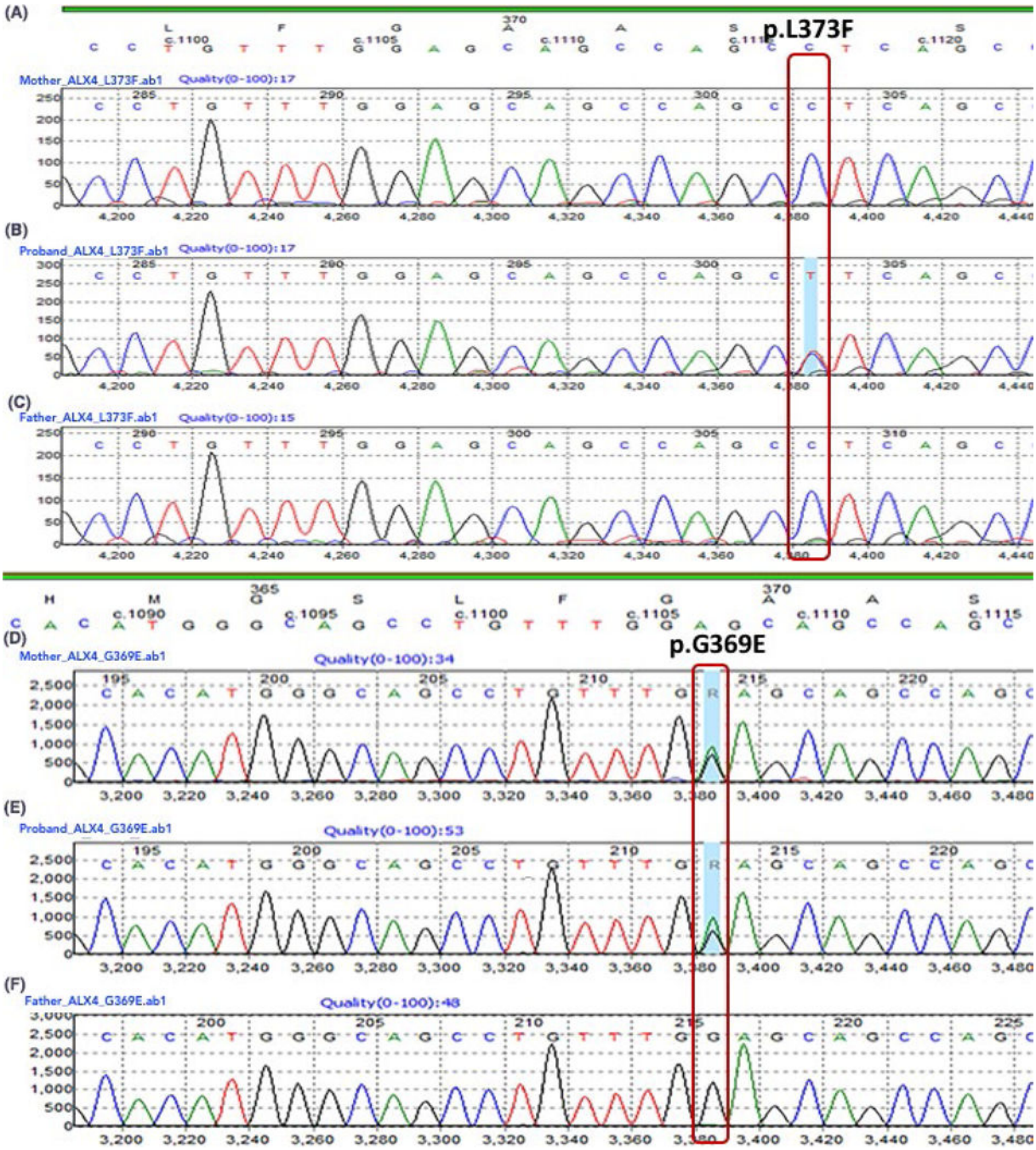

FIGURE 2.

A de novo SNV p.L373F and a maternally inherited p.G369E were identified in boys with genitourinary defects. Chromatogram indicating (A) mother of epispadic boy with a C nucleotide in position 1117. (B) Boy with epispadias with a T nucleotide together with a C nucleotide in position 1117. (C) Father of epispadic boy with a C nucleotide in position 1117. (D) Mother of boy with micropenis with an A nucleotide together with a G nucleotide in position 1106. (E) Boy with micropenis with an A nucleotide together with a G nucleotide in position 1106. (F) Father of boy with micropenis with an G nucleotide in position 1106

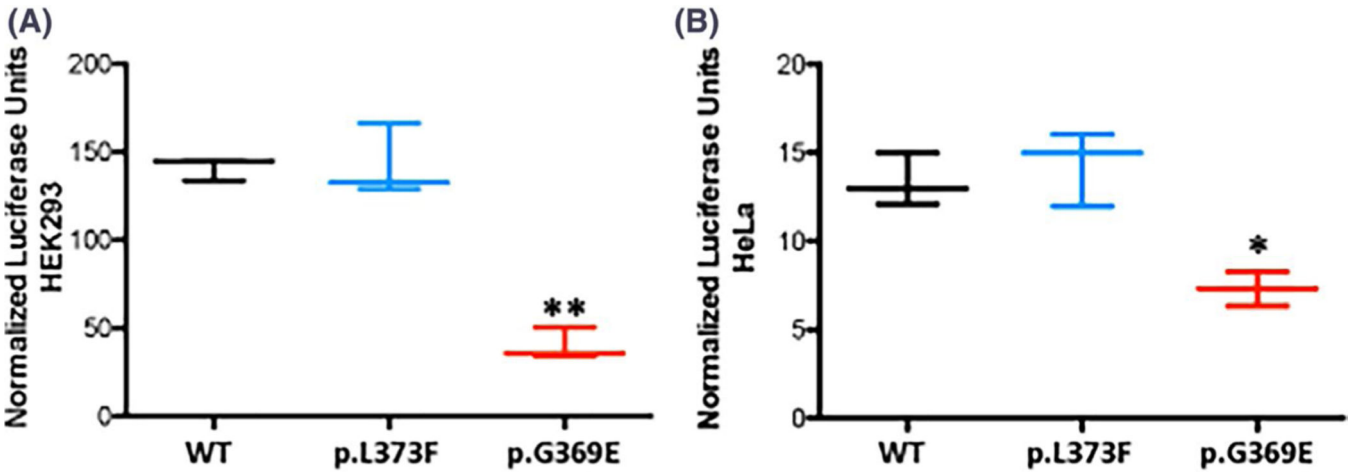

FIGURE 3.

Luciferase activities of ALX4 variants in HEK293 and HeLa cells. HEK293 (A) and HeLa cells (B) were transfected with *ALX4* expression plasmids: wild-type (WT), p.G369E and p.L373F variants. From three independent experiments in each cell line, p.G369E displayed a loss of function (HEK293: $^{**}P < .001$; HeLa: $^{*}P < .01$), while p.L373F variant did not significantly alter transcriptional efficiency relative to WT ALX4 ($P > .05$). Renilla transfection was used as an internal transfection control. The results were shown as the mean ± SEM

TABLE 1

Variants in *ALX4*(NM_021926) identified in individuals undergoing ES. Pathogenicity of variants was tested using four different prediction programs (PolyPhen2 (HumVar), SIFT-Provean, and MutationTaster). Variants predicted to be damaging are indicated in bold. Frequency of the variants in the general population of the four main databases (gnomAD, 1000G, GO-ESP, and TOPMED) is indicated. NP indicated not present in the database. Underlined phenotypes represent overlap with *Alx4* mutant mice defects. GU phenotypes are in bold type. For some probands, part of the phenotype had been solved by SNV in a different gene. All variants are heterozygous

# seen	SNV (AA) SNV (cDNA) PolyPhen2 SIFT Provean MutationTaster	Population Frequency gnomAD 1000G GO-ESP TOPMED	Phenotype
6	p.R63Q c.188G > A Benign **Damaging** Neutral **Disease causing**	T = 0.0002 T = 0.0018 T = 0.00008 T = 0.00010	**1**) prematurity, IUGR, delayed motor milestones and speech, autism spectrum disorder, intellectual disability, dysmorphic features, limb malformation, skin anomalies, **genital anomalies**, and cancer. **2**) dysmorphic features, microcephaly, proximally placed thumb, hemivertebrae, scoliosis and Tetralogy of Fallot, corpus callosum dysgenesis. **3**) developmental delay, intellectual disability, seizures, hydrocephalus, hearing loss, macrocephaly, dysmorphic features, cataract, nystagmus, and nasal duct obstruction of the left eye. **4**) microstomia, microphthalmia, increased tone of lower limbs, 2–3 toe syndactyly, cortical thumbs, talipes equinovarus, subluxation of right hip. *Two probands had been partially solved by SNV in* TTC25 *or* COL1A2.
4	p.P76T c.226C > A Benign **Damaging Deleterious Disease causing**	T = 0.0007 T = 0.0012 NP T = 0.0005	*All probands had been partially solved by SNV in* HCFC1, MED13L, COMP *or* FGFR2.
113	p.105_109del c.314_325del	=0.00001 =0.0286 =0.0264 =0.0258	*47 of the probands had been partially solved by SNV in multiple genes.* 10 individuals with GU phenotype: **1**) skeletal abnormalities (spina bifida and club foot), **omphalocele, CE, imperforate anus, ambiguous genitalia, and BEEC**. **2**) skeletal dysplasia, **neurogenic bladder,** and immunodeficiency. **3**) IUGR, prematurity, shortening of the limbs, failure to thrive, **ambiguous genitalia,** and chorioretinal atrophy. **4**) motor delay, speech delay, intellectual disability, infantile spasms, **large kidney**, and skeletal abnormalities. **5**) prematurity, mild hypotonia, dysmorphic features, bilateral retinal and iris coloboma, mild pulmonary hypertension, **kidney pelviectasis, hydronephrosis**, lumbar vertebral anomalies, skin anomalies, **genitourinary sinus**, possible bowel obstruction, and long-segment tracheal stenosis. **6**) **multiple cortical cysts in the kidneys**. **7**) motor delay, progressive muscle weakness and dystrophy, unable to close eyelids fully, myopia, joint contractures, restrictive lung function, severe muscle pain, mild degenerative arthritis in hips, progressive scoliosis, **kidney stone**. **8**) premature, dysmorphic features, postaxial polydactyly of hands bilaterally, abnormal creasing pattern of palms, broad first toes, hypoplasia of nails of toes, contractures at knees/elbows, **absent scrotum, no testes palpated, abnormal anus,bilateral dysplastic cystic kidneys,** enlarged cerebral ventricles, agenesis of corpus callosum. **9**) dysmorphia, congenital heart defects, pulmonary hypoplasia, **left cystic kidney, right echogenic kidney, andgenital anomalies**. **10**) skeletal dysplasia, short long bones, and **ambiguous genitalia**.
2	p.Q111P c.332A > C Benign Tolerated Neutral Polymorphism	G = 0.00001 NP NP NP	**1**) seizure, hypothalamic dysfunction, intellectual disability, central hypoventilation, Landau-Kleffner syndrome, adrenal hypoplasia, and auditory and visual perception disorder. **2**) prematurity, arhinia, bilateral anotia, micrognathia, hypertelorism, neonatal cardio-respiratory distress, and poor tone. Brother with bilateral ear atresia and hearing loss, micrognathia, cleft palate, bifid uvula, bilaterally cryptorchidism with a small scrotum, and congenital hypothyroidism.
3	p.S147N c.440G > A Benign Tolerated Neutral Polymorphism	T = 0.0003 T = 0.0018 T = 0.0004 T = 0.0014	**1**) delayed motor milestones and speech, dysmorphic features, tall habitus, macrocephaly, and structural brain abnormalities **2**) short stature, liver cirrhosis, copper storage disease, ichthyosis. *One proband had been solved by SNV in* ORC3.
2	p.C154S p.C154S Benign **Damaging** Neutral **Disease causing**	T = 0.0002 T = 0.0014 T = 0.0009 T = 0.0011	**1**) neutropenia, lymphopenia, recurrent pneumonia, and intermittent fever. *One proband had been solved by SNV in* KIAA2022.
6	p.E158D c.474G > C Benign Tolerated Neutral Polymorphism	G = 0.0007 G = 0.0014 NP G = 0.00002	**1**) respiratory distress, metabolic acidosis, elevated lactates, severe persistent pulmonary hypertension, obstructive sleep apnea, ankyloglossia, and oral aversion. **2**) prematurity, motor weakness, encephalopathy, generalized muscle

# seen	SNV (AA) SNV (cDNA) PolyPhen2 SIFT Provean MutationTaster	Population Frequency gnomAD 1000G GO-ESP TOPMED	Phenotype
			weakness, unsteady gait, brain atrophy, ophthalmoplegia, spasticity in the legs, and stiffness in the arms. *Four probands had been solved by SNV in* SLC52A2, ACTA1, LRBA, *or* DOCK7.
3	p.S209R c.627C > A Benign **Damaging** Neutral **Disease causing**	T = 0.0001 T = 0.0002 NP T = 0.00008	**1**) autism, ADHD, intellectual disability, sensorineural hearing loss, hypotonia, dysmorphic features, growth hormone deficiency, failure to thrive. *Two probands had been solved by SNV in* TTN *or* PYCR1.
5	p.R296Q c.887G > A Benign **Damaging Deleterious Disease causing**	T = 0.0001 T = 0.0002 T = 0.0008 T = 0.0005	**1**) IUGR, stiffness of ankles/feet, short stature, microcephaly, loss of fine motor skills, eye anomalies. **2**) delayed motor milestones and speech, intellectual disability, hearing loss, hypotonia, short stature, dysmorphic features, hyperextensibility, structural brain abnormalities, moderate hyperopia, mild astigmatism, dilated cardiomyopathy, skeletal abnormalities, and umbilical cord hernia. **3**) microtia, canal atresia, dysmorphic middle ear ossicles, T10-T11 left-sided hemivertebrae, 11 ribs, aortic coarctation, ventricular septal defect, and transverse cleft. *Two probands had been solved by SNV in* AFF2 *or* ORC3.
8	p.P306L c.917C > T Benign **Damaging Deleterious Disease causing**	A = 0.0003 NP A = 0.0006 A = 0.0011	**p.P306L has no effect *in vitro***.[38]**1**) bilateral inguinal hernia, Marcus Gunn phenomenon, partial syndactyly of the 2nd and 3rd toes, clinodactyly of 5th toes bilaterally, microcephaly. **2**) delayed motor milestones, dysmorphia, short stature, hypermobility, arthralgia, stretchy skin, fatigue, joint pain, and congenital heart disease. **3**) delayed motor milestones, delayed speech, autism, intellectual disability, seizure disorder, short stature, microcephaly, optic nerve hypoplasia, vision loss, and insomnia. **4**) dysmorphic features, eye anomalies, and congenital heart disease (Shone's complex). *Four probands had been solved by SNV in* FBLN2, ANKRD11, KCND3, PMPCA *or* DDX58.
2	p.D326N c.976G > A Benign **Damaging Deleterious Disease causing**	T = 0.00001 T = 0.0004 NP T = 0.00009	*All probands had been solved by SNV in* SLC16A2.
4	p.P327L c.980C > T Benign **Damaging** Neutral **Disease causing**	A = 0.00007 A = 0.0002 A = 0.00008 A = 0.00002	**1**) autism spectrum disorder, speech problems, macrocephaly, obesity, and epistaxis. **2**) macrocephaly because of hydrocephalus, low set ears, and structural brain abnormalities. *Two probands had been solved by SNV in* PROC *or 9.3Mb deletion of Xp22.31p22.33.*
2	p.D348N c.1042G > A Benign Tolerated Neutral **Disease causing**	T = 0.00007 NP T = 0.0002 T = 0.00009	**1**) developmental regression (loss of speech and motor skills), intellectual disability, hearing loss, hypotonia, seizure disorder, ataxia. *One proband had been solved by SNV in* PDHA1.
3	p.T362M c.1085C > T Benign Damaging Neutral **Disease causing**	A = 0.0003 NP A = 0.0002 A = 0.0001	*All probands had been solved by SNV in* KMT2D, ERF *or* SHOC2.
10	p.G369E c.1106G > A **DamagingDamaging** Neutral **Disease causing**		Detailed description Table 3.
15	p.L373F c.1117C > T Benign Tolerated Neutral **Disease causing**		Detailed description Table 2.

Italics indicate that the probands had been partially solved by SNV in a different gene.

TABLE 2

Phenotype of patients with *ALX4* heterozygous p.L373F variant. p.L373F (c.1117C > T) variant is indicated as probably damaging by MutationTaster, but benign by PolyPhen-2, PROVEAN, and SIFT. gnomAD database indicates an allele frequency of 0.0082 in African population, 0.0003 in Hispanic population, 0.00003 in European (non-Finnish) population, and 0.0004 in other population for a total frequency in all populations of 0.00079. The frequency of this variant in TOPMED was 0.0022, in GO-ESP was 0.0027, and in 1000G was 0.0026. Underlined phenotypes represent overlap with *Alx4* mutant mice defects. GU phenotypes are in bold. Patient #1 is from the BEEC cohort, and the remaining patients are from ES. Italics indicate that part of the phenotype of the patient probably has been solved by a variant in a different gene. Ethnically, H indicates Hispanic, A indicates African, C indicates Caucasian, ME indicates Middle Eastern, NS indicates not specified. Age is indicated in years

ID	Sex	Age	Ethnicity	Phenotype
1	M	6.0	NS	**Epispadias**, severe expressive, and receptive language delay.
2	M	6.01	A	**46,XY sex reversal with androgen absence**, mild developmental delay, delayed speech, short stature, failure to thrive, polyarticular juvenile rheumatoid arthritis, eosinophilic esophagitis, and skeletal dysplasia (limb bowing, tracheal issues, and abnormal scapula). Unsolved.
3	M	0.25	A	Gastroschisis, **undescended testis**, mildly dysmorphic features (downslanting palpebral fissures, low-set and posteriorly rotated ears), congenital heart defects (ventricular septal defect, patent foramen ovale). Unsolved.
4	M	5.58	NS	Epilepsy, macrocephaly, and **hydronephrosis**. Unsolved.
5	M	10.39	NS	Autism, intellectual disability, eye anomalies (left strabismus, myopia, ptosis, and astigmatism), and hearing loss. Unsolved.
6	M	4.46	NS	Asthma, recurrent pneumonia, right frontal epilepsy, and mild speech and gross motor skills. *Neurodevelopmental problems have been attributed to a ~ 4.2Mb 1q42.2q43 deletion.*
7	M	0.63	NS	Hypotonia, lactic acidemia, liver dysfunction, and right ventricular hypertrophy. This individual required NICU resuscitation because of pallor and neonatal depression and suffered neonatal death. Unsolved.
8	M	6.98	C	Language delay, intellectual disability, ADHD, seizures, anemia, and asthma. Unsolved
9	M	0.06	ME	Hypotonia, seizure disorder, upslanting palpebral fissures, microcephaly. Unsolved.
10	M	2.39	H	Developmental delay, intellectual disability, hypotonia, dysmorphic features (frontal bossing), macrocephaly, cardiomyopathy, failure to thrive, and multiple café-au-lait spots. *Intellectual disability phenotype could be associated with a* de novo *pathogenic frameshift in* NONO *c.1394dupC (p.N466fs).*. (PMID 26 571 461).
11	F	50.87	A	Cushing disease caused by pituitary adenoma. Unsolved.
12	F	0.27	NS	Congenital diaphragmatic hernia, congenital cataracts and cloudy corneas and congenital heart disease. Reported a deceased brother with congenital diaphragmatic hernia and **imperforate anus**. Unsolved.
13	F	3.57	A	Clinical suspicion of osteogenesis imperfecta type IV *Osteogenesis imperfecta could be associated with a heterozygous p.P32L variant of unknown clinical significance in* IFITM5.
14	F	10.23	NS	Static encephalopathy with global developmental delays, autism, dysmorphic features, microcephaly, anemia, and beta thalassemia trait. *Dysmorphic featurescould be associated with a pathogenic splice variant in* RAD21*, inherited from affected mother. Defects in* RAD21 *are the cause of Cornelia de Lange syndrome 4 [MIM:614701], a developmental disorder associated with facial dysmorphisms, abnormal hands and feet, growth delay, cognitive retardation, hirsutism, gastroesophageal dysfunction and cardiac, ophthalmologic, and genitourinary anomalies.*
15	F	0.87	A	Bilateral microphthalmia with small orbits and small palpebral fissures, hypoplasia of left cornea and iris, small nodule on the left anterior chest wall and a history of intrauterine growth restriction (IUGR). Unsolved.

TABLE 3

Phenotype of patients with *ALX4* heterozygous p.G369E variant. p.G369E (c.1106G > A) variant is indicated as probably damaging by MutationTaster, SIFT and PolyPhen-2 and neutral by PROVEAN. All patients are from ES. gnomAD database indicates an allele frequency of 0.0043 in Hispanic population and 0.0006 in other populations for a total frequency in all populations of 0.00055. The frequency of this variant in TOPMED was 0.0002. This variant was not identified in GO-ESP or 1000 Genome project (1000G). Underlined phenotypes represent overlap with *Alx4* mutant mice defects. GU phenotypes are in bold. Italics indicate that part of the phenotype of the patient probably has been solved by a variant in a different gene. Ethnically, H indicates Hispanic, C indicates Caucasian, NS indicates not specified. Age is indicated in years

ID	Sex	Age	Ethnicity	Phenotype
16	M	Fetus	H	**Micropenis, kidney abnormalities**, skeletal abnormalities (long bones are short and mildly thickened, mild platyspondyly, fifth finger clinodactyly), bilateral club feet, mild brachycephaly, hypotelorism, cleft lip/palate, low-set ears, communication between 4th ventricle and cisterna magna, hypoplastic vermis, bilateral choroid plexus cysts, congenital heart disease (tetralogy of Fallot with pulmonary atresia). Unsolved.
17	M	7.53	NS	Growth deficiency, feeding disorder, failure to thrive, eczema, **grade 5 vesiculo-ureteral reflux, chronic renal disease, bilateral hydronephrosis, recurrent UTI**, constipation, bilateral peripheral pulmonic stenosis, mild aortic sinotubular junction and ascending aorta hypoplasia, ischemic stroke event (sudden onset of expressive aphasia, drooling, seizures, multifocal areas of acute ischemia). Head and neck MRA showed diffuse vasculopathy. Unsolved.
18	M	6.45	NS	Developmental regression, intellectual disability, hearing loss, dystonia and mild spasticity, choreoathetoid movements, complex partial seizures, bilateral knee joint contractures, and feeding difficulty. *Bilateral knee joint contractures phenotype could be associated with a pathogenic variant in* KMT2B *inherited from his symptomatic father. Defects in* KMT2B *cause autosomal dominant childhood-onset dystonia, characterized by onset of progressive dystonia in the first decade of life.*
19	M	5.42	H & C	Developmental delay, intellectual disability, spastic quadriplegia, abnormal movements, sleep disorders. Unsolved.
20	M	3.86	NS	Hypotonia, heart murmur, mixed developmental delay, expressive and receptive language delay, clubbing of the feet, weakness, fine motor coordination problems, and failure to thrive. Unsolved.
21	F	0.08	H	Duodenal atresia with polyhydramnios, cardiac ventricular septal defect, **single pelvic kidney**, supernumerary ribs, dysmorphic features (including mild micro-retrognathia, low-set ears, upslanting palpebral fissures), and hypoplastic right thumb.*Chromosomal microarray revealed micorodeletions in 2p16.3 (0.131 Mb) involving* NRXN1 *and 1q41 (0.002 Mb) including* USH2A.
22	F	3.33	H	Developmental delay, hypotonia, Chiari malformation, hydrocephalus, left microphthalmia, coloboma, nystagmus, vision loss, distal left tibial diaphyseal fracture with osteopenia, dysphagia, gastroesophageal reflux and chronic constipation, atrial septal defect, **vesicoureteral reflux**, and dysmorphic features (synophrys, frontal bossing, high arched palate). *Dysmorphic features could be associated with a* de novo *heterozygous c.1159C > T (R387C) mutation in* RARB.
23	F	7.83	H	Developmental delay, dysmorphic features, lordosis, and short stature. Unsolved.
24	F	6.19	H	Developmental delay, congenital heart defects (patent ductus arteriosus, small atrial septal defect, peripheral pulmonic stenosis, and bicuspid aortic valve), dysmorphic features (upslanting palpebral fissures, large ears, glabellar bulging, partial synophrys with long eyelashes, long philtrum, downward angle of mouth, thin upper lip, small nose, cupid shaped mouth, and high arched palate), club foot/rocker bottom feet, capillary hemangioma, bilateral simian creases, right hand camptodactyly, truncal hypotonia, and mild peripheral hypertonia. Unsolved.
25	F	2.52	H	Delayed motor milestones, delayed speech, dystonia, hypertonia, seizure, dysmorphic features, short stature, microcephaly, failure to thrive, retinopathy congenital heart disease (ventricular septal defect, patent foramen ovale), and small bowel atresia. Brain MRI showed hypoplasia of the corpus callosum. *Neurological and craniocephalic phenotype could be associated with a homozygous c.1A > G pathogenic variant in* NDE1.