SUPPLEMENTARY MATERIALS

Missense variants in homeobox domain of PBX1 cause coracoclavicular ankylosis

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Clinical reports

Patient 1. (University of British Columbia, Vancouver, BC, Canada)

The proband was a 5-month-old male patient. Apart from clomiphene citrate use, the pregnancy was unremarkable with no teratogenic exposures. Delivery was by Caesarean section at 41 weeks 4 days gestation due to fetal deceleration. Apgar scores were 2 at 1 minute and 7 at 5 minutes with meconium aspiration and positive pressure ventilation required initially. Birth weight, length and head circumference plotted on the 50th centile. He was discharged home on day 14 of life. An inguinal hernia was noted at 2 months and respiratory arrest occurred around that time. During the work up for this, laryngomalacia with normal vocal cord movement, persistent hoarse voice, gastroesophageal reflux disease, failure to thrive, hyperextensible joints and patent ductus arteriosus (PDA) were noted. PDA was treated by coiling. He was prescribed a hip brace at night for his acetabular dysplasia. Parents were healthy, nonconsanguineous and of Punjabi Indian origin. A maternal uncle had 2 healthy children and 1 daughter with intellectual disability, very little speech at age 7 and no obvious dysmorphism. The family history was otherwise unremarkable.

The boy was seen again at age 4 years 8 months. He had been diagnosed with autism spectrum disorder the year before and had recently had bilateral orchidopexy for markedly hyper-retractile testes. He had advanced dental caries and required placement of 15 crowns under anesthesia. He continued to have diffuse joint laxity, but his voice had normalized. He had mild bilateral ptosis on ophthalmologic exam. He had global developmental delay with walking at age 2, using utensils at age 3 and no toilet training or speech besides grunting vocalizations. His receptive speech was better, and he could follow 2 step commands. Height was 101.7 cm (3rd to 15th centile), weight was 14 kg (3rd to 15th centile) and head circumference was 50.5 cm (15th to 50th centile). The head shape was mildly triangular with parietal bossing and there were sparse medial eyebrows. The nose was prominent and tubular with a mild fusiform shape and upturned tip. The philtrum was mostly smooth, grade 4/5. The ears were prominent with simple helices and a scalloped tragus on the right. The upper back was rounded in two dimensions and there was a mild upper thoracic dextroscoliosis. Borderline wide-spaced nipples were present. No hepatosplenomegaly was present. Moderate to severe joint laxity was noted. Hands showed a right simian crease, and an extra palmar crease was seen on the right, above the usual lateral transverse crease. Palmar creases were deep. Tapered fingers and mildly small finger and toenails were present.

Investigations including chromosome microarray, Fragile X, brain MRI, abdominal ultrasound, audiology, newborn screening, ammonia, lactate, calcium, phosphorus, 25-hydroxy vitamin, acylcarnitine profile, transferrin isoelectric focusing, mucopolysaccharides in urine, urine organic acids, urine purines and pyrimidines, and plasma amino acids were normal. PTH was mildly elevated at 8.9 pmol/L (normal range 1.5-7.6 pmol/L).
X-rays at 3 months showed a calcified cephalohematoma overlying the left parietal bone. Normal vertebral bodies and intervertebral disc spaces were present. There was expansion and deformity of the lateral aspect of the clavicles, bilateral vertical orientation of the coracoid processes, marked thickening and advanced ossification of the ischiopubic rami and mildly dysplastic acetabuli.
MRI of the shoulders showed they had a dysplastic appearance, encompassing excessive scapular anteversion, flared and foreshortened distal clavicles and high-riding humeral heads with mild posterior subluxation. Mild glenoid retroversion was seen, 3 to 5 degrees on the left and 8 to 10 degrees on the right.
Clinical exome sequencing at Blueprint Genetics in 2019 showed 3 variants of uncertain significance, NM_002585.4:c.844A>G, p.Asn282Asp in *PBX1*, c.407G>A, p.Ser136Asn (maternally inherited) in *SMARCA2* and c.171_180del, p.Lys57Asnfs*17 in *KLHDC10* (all hg19). None of the variants were found in gnomAD and all were expected to be damaging. Since *KLHDC10* was not associated with a known OMIM phenotype and the boy's phenotype did not match with Nicolaides-Baraitser syndrome, but *PBX1* was known to be associated with developmental delay, it was concluded this was the most likely disease-causing variant.
X-rays were circulated through the International Skeletal Dysplasia listserv and no diagnosis was obtained, although one of us (G.N.) recalled Patient 1 from his files and recognized the radiologic similarities between the two patients. One of us (G.N.) was later consulted on Patient 3 and recognized it as a third case.

Patient 2. (Chiba Child & Adult Orthopaedic Clinic, Chiba, Japan. Supplementary Figure 1A, B)

The patient was a 7-year-old girl. She was born to nonconsanguineous parents after uneventful pregnancy. Her birth weight was 2932 g (-0.2 SD). Apgar scores were 7 at 1 min and 8 at 5 minutes, respectively. Soon after birth, she was transferred to NICU for severe respiratory failure and requiring mechanical ventilation. She developed cardiac failure due to PDA and underwent ligation procedure. She was discharged at the age of one year and two months. Her developmental milestones were delayed, attained head control at 6 months,

rolling over at 8 months, walking alone at two years. Her shoulder exhibited unique shape, with both clavicles showing thick and short, representing projective scapulae.
Whole exome sequencing was performed using a SureSelect Human All Exon V5 + UTRs Kit (Agilent Technologies), followed by 101-bp paired-end sequencing on a HiSeq 2500 (Illumina), according to the manufacturers' instructions. Sequencing data were analyzed with the Burrows-Wheeler Alignment SAMTools, Picard, the Genome Analysis Toolkit, and SnpEff for variant annotation. Copy number variation analysis was performed using the log2-ratio of read depth on each exon as described previously (Enomoto et al., 2022). We identified a novel variant, c.850T>A, p.(Phe284Ile) in *PBX1* (NM_002585.4). Sanger sequencing confirmed that the variant was de novo. The variant is not included in the public databases gnomAD, jMorp, ClinVar, Human Genetic Variation Database, or the Human Genome Mutation Database 2022.3. The highest CADD (Combined Annotation Dependent Depletion) score was 26.0. The variant is classified as likely pathogenic according to the American College of Medical Genetics and Genomics standards and guidelines for the interpretation of sequence variants (PS2, PM2, PP3, PP4) (Richards et al., 2015). No other variant or CNV that could be associated with her condition was detected in this study.

Patient 3. (Kanagawa Children's Medical Center, Yokohama, Japan. Supplementary Figure 1C, D)

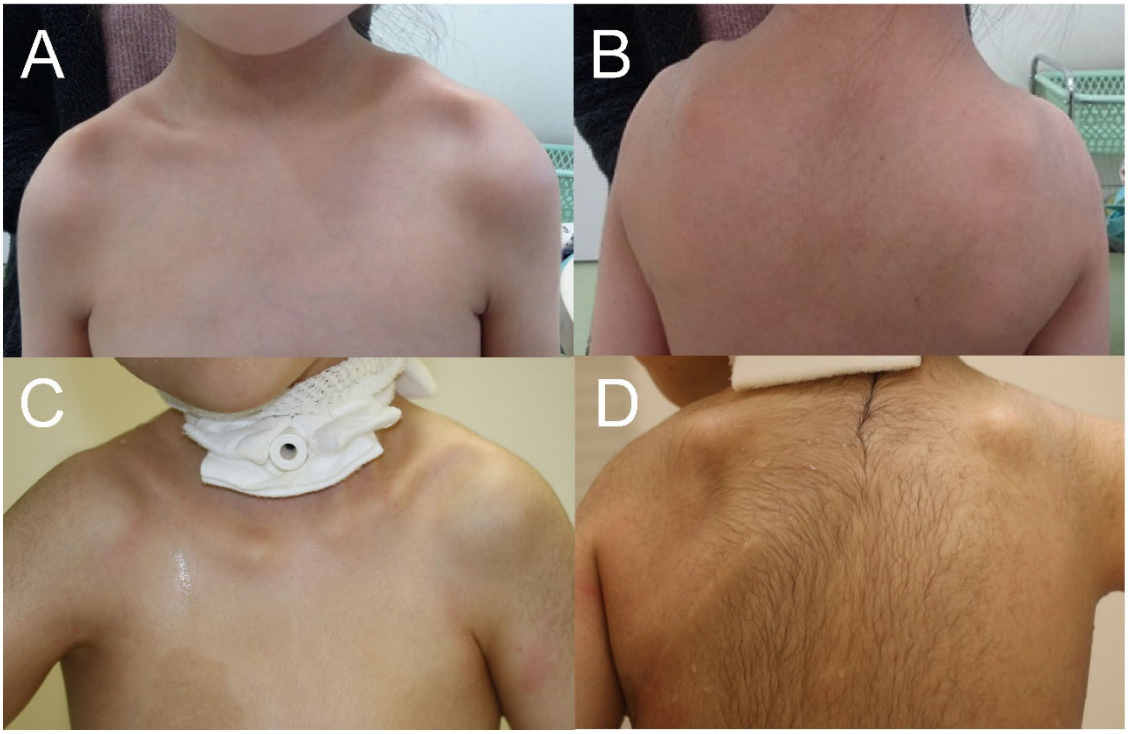
The proband was a 4-year-old female patient. The pregnancy was complicated with increased nuchal translucency from the first trimester. Amniocentesis revealed a normal female karyotype. At 30 weeks of gestation, the stomach bubble was not visible, and polyhydramnios was noted, suggesting possible esophageal atresia. She was born at 34 weeks and 5 days of gestation to a 27-year-old mother via cesarean section due to non-reassuring fetal status. Her birth weight and length were 1982 g (-0.7 SD) and 43.5 cm (-0.6 SD), respectively. After birth, she had respiratory distress requiring surfactant therapy followed by mechanical respiratory support. She showed hypoplastic thoracic cage and laryngomalacia but no esophageal atresia. Echocardiography showed left ventricular heart failure, patent ductus arteriosus (PDA), pulmonary hypertension, persistent left superior vena cava (PLSVC), and patent foramen ovale (PFO). PDA was surgically ligated at twenty days. Because of recurrent acute respiratory failure episodes, she underwent tracheostomy and supported by a mechanical ventilator after the age of two years. Her developmental milestones were severely delayed. She was able to stand with support at age three but unable to walk by four years without support. She had severe feeding difficulty and underwent gastrostomy from the age of four. Ultrasonography revealed a large gallstone

but standard shape and size in the renal pelvis and calyces of bilateral kidneys. Her dysmorphic features included a cleft soft palate, auricular hypoplasia, and hypoplastic nipples. Ophthalmologic investigation revealed choroid plexus cyst and glaucoma. Her thorax and shoulders were slim. She presented hunchback posture when she was sitting. Her scapula was anterior rotated and lateral displacement. Her hip joint was flexion and medial displacement.

A systematic bone survey during the neonatal period revealed skeletal dysplasia, including a fusion (ankylosis) between the clavicle and coracoid process of the scapula, premature closure of the ischiopubic synchondrosis and broad ischia, and mandible-like hyoid arch. She showed facial dysmorphism including arched eyebrows, hypertelorism, epicanthus, bulbous nose, prominent Cupid’s bow, and prominent antihelix of the ears. She also had sloping shoulders, as did patient 2. No finger deformities were observed.

Clinical information was collected after obtaining written informed consent from the patient's family. This study was approved by the institutional review board of Kanagawa Children's Medical Center. According to the manufacturer's instructions, genomic DNA was extracted from the patient's peripheral blood and both parents using a QIAcube kit (QIAGEN, Hilden, Germany). Whole exome sequencing was performed for both the affected patient and her parents. Genomic DNA captured by Agilent SureSelect Human All Exon V6 (Agilent Technologies, Santa Clara, CA) was sequenced on a HiSeq2500 (Illumina, Inc. San Diego, CA) with 151-base pair paired-end reads. We identified a heterozygous variant NM_002585.4:c.869G>T: p.(Arg290Leu) (hg19) of *PBX1*. Sanger sequencing confirmed that the variant was a de novo event.

Supplementary Figure



Supplementary Figure 1. Patient 2 (A, B) at age 7 years and patient 3 (C, D) at age 6 years. The clavicle was thickened and shortened. The scapula was pulled anteriorly and displaced by the fusion of the coracoid process of the scapula and the coracoid tubercle of the clavicle, or coracoclavicular ankylosis (A, B, C, D).

Supplementary Table 1. Genetic and clinical features of the present patients and the previously reported cases with a missense variant in homeobox domain of PBX1

	Present study			Ruscitt et al. (2022)
	Patient 1	Patient 2	Patient 3	
Variant (NM_002585.4)	c.844A>G	c.850T>A	c.869G>T	c.868C>T
Protein change (NP_002576.1)	p.Asn282Asp	p.Phe284Ile	p.Arg290Leu	p.Arg290Trp
Genome position (GRCh38:NC_000001.11)	g.164811996A>G	g.164812002T>A	g.164812021G>T	g.164812020C>T
CADD (v1.6)	27.6	26	31	29
REVEL	0.937	0.984	0.961	0.867
ACMG variant classification	PS1+PM1+PM2+PP3+PP4 Likely pathogenic	PS1+PM1+PM2+PP3 +PP4 Likely pathogenic	PS2+PM1+PM2+PM5+PP 3+PP4+P5 Pathogenic	PS2+PM1+PM2+PP3+PP4+PP5 Likely pathogenic
Sex	Male	Female	Female	Male
Age at latest investigation	7	7	6	Day 4
Craniofacial characteristics				
Eye abnormality	Ptosis	-	Choroid plexus cyst, glaucoma	-
Cleft palate	-	-	Cleft soft palate	-
Ear anomaly	Simple helices and scalloped tragus on the right	-	Prominent inferior crus of antihelix	Low-set posterior ly rotated ears
Facial dysmorphisms	Sparse medial eyebrows, upturned nares, smooth philtrum	-	Mask-like face	Micrognathia
Cardiopulmonary				
	Meconium aspiration requiring ventilation	Respiratory failure	Pulmonary hypoplasia, peristent pulmonary hypertension of the newborn.	Pulmonary hypertension and total anomalous pulmonary venous return, and pulmonary dysmorphisms
Patent ductus arteriosus (PDA)	+	+	+	+
Laryngomalasia/tracheomalasia	+	+	+ (Severe)	NA
CAKUT				
Renal hypoplasia/dysplasia	-	-	-	Double renal pelvis with double ureter on the right side
Genital anomaly	-	-	-	-
Skeletal				
Coracoclavicular ankylosis	+	+	+	NA
Thick clavicles	+	+	+	Cleidosternal dysostosis
Premature ossification or absence of the ischiopubic synchonrosis	+	+	+	NA
Hip dislocation/subluxation	-	-	-	NA
Lumber lordosis	-	-	-	-
Spina bifida	-	-	-	NA
Rib abnormalities	-	-	-	NA
Other skeletal anomalies	-	-	-	-
Skin	-	-	Hypoplastic nipples	-
Deafness	-	-	+	NA
Neurodevelopment	Autism spectrum disorder, global developmental delay with walking at 2 years	Mild developmental delay	Severely delayed, walking at 5 years, autism spectrum disorder	NA
Brain MRI	Normal	NA	Hypoplastic corpus callosum	-
Gastrointestinal	Gastroesophageal reflux	-	Gastrostomy due to feeding difficulty	Hepatomegaly, colonic duplication and malrotation of the appendix
Joint laxity	+	+	+	NA
Other			Polyhydramnios	Polyhydramnios

Supplementary Table 1. Genetic and clinical features of the present patients and the previously reported cases with a missense variant in homeobox domain of PBX1 (Continued)

Kammoun et al. (2018)	Slavotinek et al.2017			Eozenou et al. (2019)	Bartolli-Avella et al. (2021)
	Patient 4	Patient 5	Patient 8		
c.700C>T	c.701G>C	c.704G>A	c.704G>C	c,704G>T	c.836A>G
p.Arg234Trp	p.Arg234Pro	p.Arg235Gln	p.Arg235Gln	p.Arg235Gln	p.Gln279Arg
g.164799888C>T	g.164799889G>C	g.164807544G>A	g.164807544G>C	g.164807544G>T	g.164807676A>G
25.2	34	33	31	31	32
0.751	0.925	0.854	0.868	0.899	0.953
PS2+PM1+PM2+PP3+PP4 Likely pathogenic	PS2+PS3+PM1+PM2+PP3+PP4 Pathogenic	PS2+PS3+PM1+PM2+PP3+PP4 Pathogenic	PS2+PS3+PM1+PM2+PP3+PP4 Pathogenic	PS2+PS3+PM1+PM2+PP3+PP4 Pathogenic	PS2+PM1+PM2+PP3+PP4 Likely pathogenic
NA	Female	Male	Famale (46,XY)	Female (46,XY)	Male
NA	2	22 M	3 M	12.3	NA
NA	-	-	-	-	-
NA	-	-	-	-	-
NA	-	Microtia with prominent lobules	-	-	
NA	-	Prominent nuchal folds, micrognathia	-	-	Dolicocephaly, narrow forehead, prominent nose, deeply set eye, highly arched eyebrow
NA	Perinatal asphyxia with chronic lung disease, pulmonary artery hypertension	Lung hypoplasia, paralyzed right diaphragm, persistent pulmonary hypertension	Respiratory insufficiency with hypercapnia	-	Atral septal defect, coarctation of aorta, neonatal respiratory distress, Recurrent upper respiratory tract infections, pulmonary artery stenosis
NA	+	+	-	-	+
NA	-	NA	-	-	-
NA	- (Pyelocaliectasia +)	-	-	-	
NA	-	-	46,XY gonadal dysgenesis	46,XY gonadal dysgenesis	Cryptorchidism
NA	-	-	-	-	NA
NA	-	-	-	-	NA
NA	-	-	-	-	NA
NA	-	-	-	-	NA
NA	-	-	-	-	NA
NA	-	-	-	-	NA
NA	-	-	-	-	NA
NA	-	-	-	Radiocubital synostosis	Barrel-shaped chest
NA	-	-	-	-	-
NA	-	-	-	-	-
NA	Global developmental delay, walking with assistance at 2 years	-	-	-	Muscle hypotponia, hyperreflexia, delay gross motor development
NA	NA	NA	NA	NA	NA
NA	Gastrostomy due to gastrointestinal reflux	-	Difficulty with oral intake and requires tube feeding	NA	Achalasia, esophageal stenosis
NA	-	-	-	-	+
Diaphragmatic hernia	Polyhydramnios		Polyhydramnios, inguinal hernia		NA

Supplementary Table 2. Reported variants and the references cited in Figure 2.

Variant (NM_002585.4)	Protein change (NP_002576.1)	References
c.145C>T	p.Gln49*	Tran Mau-Them F, Moutton S, Racine C, Vitobello A, Bruel AL, Nambot S, et al. Second-tier trio exome sequencing after negative solo clinical exome sequencing: an efficient strategy to increase diagnostic yield and decipher molecular bases in undiagnosed developmental disorders. Hum Genet. 2020;139:1381-90.
c.234del	p.Phe78Leufs*	Li J, Wang L, Yu P, Shi L, Zhang K, Sun ZS, et al. Vitamin D-related genes are subjected to significant de novo mutation burdens in autism spectrum disorder. Am J Med Genet B Neuropsychiatr Genet. 2017;174:568-77.
c.263C>T	p.Thr88Ile	Fromer M, Pocklington AJ, Kavanagh DH, Williams HJ, Dwyer S, Gormley P, et al. De novo mutations in schizophrenia implicate synaptic networks. Nature. 2014 ;506(7487):179-84.
c.319C>T	p.Arg107Try	Arts P, Garland J, Byrne AB, Hardy TSE, Babic M, Feng J, et al. Paternal mosaicism for a novel PBX1 mutation associated with recurrent perinatal death: Phenotypic expansion of the PBX1-related syndrome. Am J Med Genet A. 2020;182:1273-7.
c.320G>C	p.Arg107Pro	Pode-Shakked B, Barel O, Singer A, Regev M, Poran H, Eliyahu A, et al. A single center experience with publicly funded clinical exome sequencing for neurodevelopmental disorders or multiple congenital anomalies. Sci Rep. 2021;11:19099.
c.400dup	p.Ala134Glyfs*	Safgren SL, Olson RJ, Pinto E Vairo F, Bothun ED, Hanna C, Klee EW, et al. De novo PBX1 variant in a patient with glaucoma, kidney anomalies, and developmental delay: An expansion of the CAKUTHED phenotype. Am J Med Genet A. 2022;188:919-25.

c.413_419delGGGCAGG	p.Gly138Valfs*	Riedhammer KM, Siegel C, Alhaddad B, Montoya C, Kovacs-Nagy R, Wagner M, et al. Identification of a Novel Heterozygous *De Novo* 7-bp Frameshift Deletion in *PBX1* by Whole-Exome Sequencing Causing a Multi-Organ Syndrome Including Bilateral Dysplastic Kidneys and Hypoplastic Clavicles. Front Pediatr. 2017;5:251.
c.428delA	p.Asn143Thrfs*	Heidet L, Morinière V, Henry C, De Tomasi L, Reilly ML, Humbert C, et al. Targeted Exome Sequencing Identifies *PBX1* as Involved in Monogenic Congenital Anomalies of the Kidney and Urinary Tract. J Am Soc Nephrol. 2017;28:2901-14.
c.511-2A>G		Heidet L, Morinière V, Henry C, De Tomasi L, Reilly ML, Humbert C, et al. Targeted Exome Sequencing Identifies PBX1 as Involved in Monogenic Congenital Anomalies of the Kidney and Urinary Tract. J Am Soc Nephrol. 2017;28:2901-14.
c.550C>T	p.Arg184*	Heidet L, Morinière V, Henry C, De Tomasi L, Reilly ML, Humbert C, et al. Targeted Exome Sequencing Identifies *PBX1* as Involved in Monogenic Congenital Anomalies of the Kidney and Urinary Tract. J Am Soc Nephrol. 2017;28:2901-14.
c.551G>C	p.Arg184Pro	Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26:4849-60.
c.567del	p .Arg190Glyfs*	Morichi S, Suzuki S, Kasuga A, Ishida Y, Yamanaka G, Kashiwagi Y, et al. A New Pathogenic Variant of CAKUTHED Diagnosed Based on Intellectual Disability. Indian J Pediatr. 2020;87:480-1.
c.626G>C	p.Ser209Thr	Takata A, Miyake N, Tsurusaki Y, Fukai R, Miyatake S, Koshimizu E, et al. Integrative Analyses of De Novo Mutations Provide Deeper Biological Insights into Autism Spectrum Disorder. Cell Rep. 2018;22:734-47.

c.661G>T	p.Glu221*	Riedhammer KM, Braunisch MC, Günthner R, Wagner M, Hemmer C, Strom TM, et al. Exome Sequencing and Identification of Phenocopies in Patients With Clinically Presumed Hereditary Nephropathies. Am J Kidney Dis. 2020;76:460-70.
c.671T>A	p.Met224Lys	Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26:4849-60.
c.680G>C	p.Arg227Pro	Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26:4849-60.
c.701G>C	p.Arg234Pro	Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26:4849-60.
c.700C>T	p.Arg234Trp	Kammoun M, Souche E, Brady P, Ding J, Cosemans N, Gratacos E, et al. Genetic profile of isolated congenital diaphragmatic hernia revealed by targeted next-generation sequencing. Prenat Diagn. 2018;38:654-63.
c.704G>A	p.Arg235Gln	Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26:4849-60.

c.778T>C	p.Tyr260His	Deciphering Developmental Disorders Study. Prevalence and architecture of de novo mutations in developmental disorders. Nature. 2017;542(7642):433-8.
c.783dup	p.Ser262Glu*	Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26:4849-60.
c.836A>G	p.Gln279Arg	Bertoli-Avella AM, Beetz C, Ameziane N, Rocha ME, Guatibonza P, Pereira C, et al. Successful application of genome sequencing in a diagnostic setting: 1007 index cases from a clinically heterogeneous cohort. Eur J Hum Genet. 2021;29:141-53.
c.862C>T	p.Arg288*	Slavotinek A, Risolino M, Losa M, Cho MT, Monaghan KG, Schneidman-Duhovny D, et al. De novo, deleterious sequence variants that alter the transcriptional activity of the homeoprotein PBX1 are associated with intellectual disability and pleiotropic developmental defects. Hum Mol Genet. 2017;26:4849-60.
c.868C>T	p.Arg290Trp	Ruscitti F, Cerminara M, Iascone M, Pezzoli L, Rosti G, Romano F, et al. An example of parenchymal renal sparing in the context of complex malformations due to a novel mutation in the PBX1 gene. Birth Defects Res. 2022;114:674-81.
c.1142C>T	p.Thr381Ile	Li J, Wang L, Yu P, Shi L, Zhang K, Sun ZS, et al. Vitamin D-related genes are subjected to significant de novo mutation burdens in autism spectrum disorder. Am J Med Genet B Neuropsychiatr Genet. 2017;174:568-77.