Published in final edited form as:

Am J Med Genet A. 2018 November ; 176(11): 2404–2418. doi:10.1002/ajmg.a.40482.

Warsaw Breakage Syndrome: Further Clinical and Genetic Delineation

Ebba Alkhunaizi^{1,2}, Ranad Shaheen³, Sanjay Kumar Bharti⁴, Ann M. Joseph-George⁵, Karen Chong¹, Ghada M H Abdel-Salam⁶, Mohammed Alowain⁷, Susan I Blaser⁸, Blake C Papsin⁹, Mohammed Butt¹⁰, Mais Hashem³, Nicole Martin¹, Ruth Godoy¹, Robert M. Brosh Jr.⁴, Fowzan S Alkuraya^{#3,11,12}, and David Chitayat^{#1,2}

¹The Prenatal Diagnosis and Medical Genetics Program, Department of Obstetrics and Gynecology, Mount Sinai Hospital, University of Toronto, Toronto, Ontario, Canada

²Division of Clinical and Metabolic Genetics, Department of Pediatrics, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada

³Department of Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia

⁴Laboratory of Molecular Gerontology, National Institute on Aging, National Institutes of Health, NIH Biomedical Research Center, 251 Bayview Blvd, Baltimore, MD 21224, USA

⁵Cytogenomics Laboratory, Division of Genome Diagnostics, Department of Pediatric Laboratory Medicine, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada

⁶Department of Clinical Genetics, Human Genetics and Genome Research Division, National Research Centre, Cairo, Egypt

⁷Department of Medical Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia

⁸Division of Neuroradiology, Department of Diagnostic Imaging, The Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada

⁹Department of Otolaryngology - Head & Neck Surgery, The Hospital for Sick Children, University of Toronto, Toronto, ON, Canada

¹⁰Department of Radiology, King Abdulaziz University Hospital, King Saud University, Riyadh, Saudi Arabia

¹¹Department of Anatomy and Cell Biology, College of Medicine, Alfaisal University, Riyadh, Saudi Arabia

¹²Saudi Human Genome Program, King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia

These authors contributed equally to this work.

* **Correspondence to:** Dr. David Chitayat, The Ontario Hydro Generation Building, 700 University Av., 3rd floor, Rm. 3-709, Toronto, Ontario, Canada, M5G 1Z5. Phone: 416-586-4523; Fax: 416-586-4723; david.chitayat@sinahealthsystem.ca.

Conflict of interest: none declared.

Abstract

Warsaw breakage syndrome (WBS) is a recently recognized *DDX11*-related rare cohesinopathy, characterized by severe prenatal and postnatal growth restriction, microcephaly, developmental delay, cochlear anomalies and sensorineural hearing loss. Only seven cases have been reported in the English literature, and thus the information on the phenotype and genotype of this interesting condition is limited.

We provide clinical and molecular information on five additional unrelated patients carrying novel bi-allelic variants in the *DDX11* gene, identified via whole exome sequencing. One of the variants was found to be a novel Saudi founder variant. All identified variants were classified as pathogenic or likely pathogenic except for one which was initially classified as a variant of unknown significance (VOUS) (p.Arg378Pro). Functional characterization of this VOUS using heterologous expression of wild type and mutant *DDX11* revealed a marked effect on protein stability, thus confirming pathogenicity of this variant.

The phenotypic data of the seven WBS reported patients were compared to our patients for further phenotypic delineation. Although all the reported patients had cochlear hypoplasia, one patient also had posterior labyrinthine anomaly. We conclude that while the cardinal clinical features in WBS (microcephaly, growth retardation and cochlear anomalies) are almost universally present, the “breakage” phenotype is highly variable and can be absent in some cases. This report further expands the knowledge of the phenotypic and molecular features of WBS.

Keywords

DDX11; Warsaw breakage syndrome; Cohesinopathy; Exome sequencing; Microcephaly; Growth restriction; Cochlear anomalies; Hearing loss

INTRODUCTION

Warsaw breakage syndrome (WBS) (MIM# 601150) is a recently described as an autosomal recessive cohesinopathy characterized by severe prenatal and postnatal growth retardation, severe microcephaly, sensorineural hearing loss, cochlear anomalies, intellectual disability, abnormal skin pigmentation and other less common congenital anomalies. The condition is caused by bi-allelic variants in the *DDX11* gene, a member of the conserved Superfamily 2 (SF2) Iron-Sulfur (Fe-S) cluster DNA helicases [Hirota and Lahti, 2000; Wu et al., 2009]. *DDX11* plays a vital role in normal cohesion of sister chromatids, chromosomal maintenance and stability [Parish et al., 2006; Wu et al., 2009]. Hence, the possibility of increased cancer risk in heterozygous carriers of *DDX11* mutations was raised although not proven [van der Lelij et al., 2010a].

To date, seven patients with WBS have been reported with each having a different bi-allelic variant in the *DDX11* gene [van der Lelij et al., 2010a; Capo-Chichi et al., 2013; Bailey et al., 2015; Eppley et al., 2017]. The majority of WBS patients were diagnosed through whole exome sequencing (WES) likely due to the novelty and rarity of the condition [Capo-Chichi et al., 2013; Eppley et al., 2017]. The diagnosis can also be supported by the cytogenetic findings of increased chromosomal breakage and sister chromatid repulsion [van der Lelij et

al., 2010a; Capo-Chichi et al., 2013; Bailey et al., 2015; Eppley et al., 2017]. The knowledge gathered on the underlying pathogenetic mechanism is evolving, and the distinctive combined cytogenetic findings bring an overlapping phenotype with Fanconi anemia (MIM# 227650), Nijmegen breakage syndrome (MIM# 251260), and Robert syndrome (MIM# 268300) [van der Lelij et al., 2010a].

In our study, we elucidate the clinical manifestations and the results of investigations performed on five affected patients with five novel bi-allelic *DDX11* gene variants, and compare the findings on our patients to the other seven cases reported in the English literature. While four of these five novel bi-allelic variants can readily be classified as pathogenic/likely pathogenic, we investigated one homozygous variant of unknown significance (VOUS) using functional assays and we report the results of these analyses. Finally, we reviewed the available family histories of the affected patients to assess the possibility of increased cancer risk in heterozygous carriers [van der Lelij et al., 2010a].

PATIENTS AND METHODS

Patients' ascertainment:

We ascertained five affected individuals with a molecular diagnosis of WBS identified via WES. Three subjects had WES as part of IRB-approved research protocol and appropriate informed consent was obtained (KFSRHC RAC# 2121-053 and RAC# 2080-006), while the other two had clinical WES performed as part of routine clinical care. Informed consent for photographs and publication was obtained from their legal guardians, according to the protocol approved by Mount Sinai Hospital in Toronto, Canada for patients 1 and 2 and King Faisal Specialist Hospital & Research Centre in Riyadh, Saudi Arabia for patients 3, 4 and 5. The clinical information is summarized in Table I.

Patient 1: This male patient was born to non-consanguineous parents of Croatian / Italian descent. Family history was non-contributory aside from a maternal uncle who died in his early 20's of lymphoma. The pregnancy was complicated by intrauterine growth restriction (IUGR), and delivery was at 37 weeks gestation and uncomplicated. The birth weight was 2154 g (−2.3 SD) and although other birth growth parameters were not available, his parents recall that he had a small head circumference. After birth, he failed newborn hearing screen bilaterally and temporal bone imaging showed incomplete partition of the cochlea, tapering of the ductus reunions, primitive anlage of the lateral semicircular canal (absent bone island of the lateral semicircular canal) and bilateral small cochlear nerves (Fig.1a-d). Brain MRI showed microcephaly vera with simplification of the gyri, normal ventricles, and normal midline structures (Fig.2a-a¹). During the first two years of life, he was noted to have short stature, low weight, microcephaly and sensorineural hearing loss.

At 18 months of age, he had a significant speech delay and cochlear implants were placed. At 4 years of age he had 30 words and his motor milestones were appropriate for age. At 7 years of age, he started having tonic clonic seizures and is on clonazepam 1 mg daily with moderate effect. Physical examination at 20 months showed that his weight was 7.7 kg (−4.9 SD), his height was 68 cm (−4.8 SD), and his head circumference was 40.5 cm (−5.6 SD). He had a small face and thin body habitus, bilateral epicanthal folds, a large nose, a

prominent upper lip, a retrognathia and small cupped ears (Fig.3A). He had 5th finger clinodactyly, hypoplastic finger nails and 2/3 toe syndactyly, bilaterally. His cardiovascular and abdominal examinations were normal. He had normal male external genitalia. His back was straight and his skin showed neither hyper- nor hypopigmented lesions. He had normal strength and tone, and his ophthalmology examination showed no abnormalities.

DNA analysis for *GJB2*, *GJB6*, as well as *MTRNR1* and *MTTS1* mitochondrial genes, associated with hearing loss showed no detectable causative variant. He also had a normal chromosomal microarray analysis (aCGH) and negative H19DMR methylation and 11p15 gene dosage studies for Russell-Silver syndrome.

Patient 2: This was the second live-born of a consanguineous Pakistani union. Family history was significant for five previous miscarriages in the context of normal parental karyotypes. The mutual grandfather to both parents was diagnosed with kidney cancer at 66 years of age. Additionally, a mutual half-cousin was diagnosed with cancer of an unknown site in his mid-40.

The couple was seen prenatally for fetal ultrasound findings of brain dysgenesis and IUGR. Fetal brain MRI at 28 weeks showed delayed fissuration, sulcation, operculation and head circumference consistent with 19.6 weeks gestation.

The patient was born at 38 weeks with birth weight of 1500 gram (−3.3 SD), length of 39.5 cm (−4.2 SD) and head circumference of 26 cm (−4.3 SD). Hearing test showed a bilateral profound hearing loss, and temporal bone CT scan showed severely hypoplastic cochlea, bilateral absence of the bony cochlear nerve canal but normal posterior labyrinth. MRI of the inner ear re-demonstrated these findings and confirmed absence of the cochlear nerve bilaterally (Fig.1i-l). Brain MRI showed microcephaly vera, a gyral simplification with the appropriate myelination for age, normal ventricles and no midline structures abnormalities (Fig.2b-b¹). Echocardiography showed small atrial septal defect (ASD) and a large patent ductus arteriosus (PDA) with bidirectional shunting. He had hypospadias that was corrected surgically. Ophthalmology evaluation showed strabismus and myopia. Abdominal ultrasound was normal. He underwent placement of an auditory brainstem implant with no major improvement. There were no recurrent infections. Developmentally, he has a moderate intellectual disability. He walked at 18 months but he is still non-verbal and can communicate with sign language.

At 9 years of age, physical examination showed weight of 29.7 kg (50 - 75th centile), height of 123.3 cm (3rd - 10th centile), and head circumference of 43 cm (−7.4 SD). He had brachycephaly, short forehead, triangular and small face with hypoplasia of the supraorbital ridges and deep-set eyes. His nasal bridge was depressed and he had a prominent nose with overhanging columella, narrow nares and a short philtrum. Both ears were small, cupped and low-set. He had retrognathia and full lips (Fig.3B). He had brachydactyly and proximal insertion of both thumbs. There was left clinodactyly of the 5th toe, and the 5th toe was overlapping the 4th toe, bilaterally. On the right, the 4th toe was overlapping the 3rd toe. The 5th toe was proximally inserted, and there was 2/3 toes partial syndactyly (Fig.4A). His genitalia showed hypoplastic scrotum and both testes were descended. There were two Café-

au-lait spots measuring 1.7×0.2 cm at the left costal margin and 0.5×0.5 cm on the right lower leg. Neurological examination showed generalized hypotonia and brisk deep tendon reflexes.

Previous genetic testing showed normal aCGH, karyotype and *PCNT* sequencing for microcephalic osteodysplastic primordial dwarfism.

Patient 3: This Saudi girl was born at 33 weeks to a first-cousin union. The parental family histories were non-contributory. The pregnancy was complicated with IUGR and at birth, she was noted to have severe talipes equinovarus and ventricular septal defect (VSD). The first two years were marked by a significant severe failure to thrive and microcephaly. Physical examination at 2 years and 3 months of age showed weight of 9.2 kg (-2.8 SD), height of 69.5 cm (-5.9 SD), and head circumference of 38 cm (-6.7 SD). She had facial dysmorphism with small face, beaked nose with flat nasal tip, depressed nasal bridge, full lips and epicanthal folds (Fig.3C). She also had brachydactyly, clinodactyly of the 5th fingers, bilateral talipes equinovarus and skin pigmentation and eczema. There were bilateral clinodactyly of the 5th toe and proximal insertion of the 5th and 4th toes on the right (Fig. 4B). Her hearing assessment revealed severe bilateral sensorineural hearing loss. Her gross and fine motor and social milestones at this stage were appropriate for age but she had severe expressive language delay which did not improve with hearing aids.

Her most recent assessment was at 6 years of age and showed weight of 12 kg (-3.6 SD), height of 91 cm (-4.8 SD) and head circumference of 40 cm (-8.7 SD). She was noted to have recurrent ear discharges and chronic tonsillitis.

Her chromosomal microarray (aCGH) was normal and female. Temporal bone CT scan demonstrated hypoplasia of the cochlea, including the basal turn and cochlear nerve canal. The lateral semicircular canal bone island is hypoplastic and the vestibule is enlarged. The posterior limb of the superior semicircular canal is absent. The posterior semicircular canal maintains a fetal shape (anlage) with absence of a posterior semicircular canal bone island. The findings are symmetrical (Fig.1e-h).

Patient 4: This Saudi boy was born at term to a first-cousin union. Their family histories were non-contributory. The pregnancy was complicated with IUGR of an unknown etiology. His birth weight was 1,200 g (-3.8 SD) and his other birth growth parameters were not available but a small head was noted. After birth, hearing test via otoacoustic emission was suggestive of abnormal cochlear outer hair cell function. Visual reinforcement audiometry revealed severe-to-profound hearing loss with no response to 1 kilohertz or higher frequencies.

At 3 years of age, he was delayed and on Bayley scale, his mental age was consistent with 19 months of age. His gross and fine motor milestones were appropriate for age but a severe speech delay was noted. He had mild attention deficit-hyperactivity disorder (ADHD).

At 5 years of age, his physical examination showed weight of 13.2 kg (-3.9 SD), height of 103 cm (-2.2 SD), and head circumference of 42 cm (-6.5 SD). He appeared small with a thin body habitus. He had a triangular and small face, large nose, thin eyebrows,

downslanting palpebral fissures, pointed chin and retrognathia. He had bilateral clinodactyly of the 5th finger. His chest, cardiovascular, abdominal, CNS, and skin examination were unremarkable.

At 10 years of age, he had 10-15 words, understood simple commands and attended a special school. He had no recurrent infections. Brain MRI showed left frontal side flattening and decrease in the sulcal pattern, suggestive of focal lissencephaly. Skeletal survey at the age of 3 years showed triangular skull and premature fusion of the cranial sutures. CT scan of the temporal bone showed a small right cochlea and the MRI demonstrated cochlear hypoplasia, lack of cochlear nerve, but normal semicircular canals and vestibule (Fig.1m-p). Karyotype and aCGH were normal. Abdominal ultrasound was unremarkable.

Patient 5: This patient was the second-born child of first cousin parents of Egyptian descent. Their family histories revealed a brother with progressive cerebellar atrophy and mild intellectual disability, and there was no family history of cancer.

No prenatal care was provided. He was born at 38 weeks gestation by normal vaginal delivery, with a birth weight of 750 g (−3 SD) and the birth length and head circumference were not recorded but reported as small. Hearing test and auditory brain response showed a profound sensorineural hearing loss. At 14 months of age, he had motor and language delay. He sat at 9 months and walked at 3 years. During infancy and early childhood, he had severe failure to thrive and multiple episodes of infections, including recurrent otitis media.

On physical examination at 14 months his weight was 5.5 kg (−4.6 SD), his length 65 cm (−4.2 SD) and his head circumference 36 cm (−6.6 SD). He had dysmorphic facial features with a triangular and small face, receding forehead, prominent eyes, beaked nose with downturned tip and hypoplastic nostrils, short philtrum, thin lips, posteriorly rotated malformed ears and retrognathia (Fig.3D). He had clinodactyly of the 3rd, 4th, and 5th toes and 2nd toe overlaps the 3rd toe, bilaterally (Fig.4C). He had hypoplastic scrotum with bilateral undescended testes and his skin was normal with neither hyper- nor hypopigmented macules.

Follow up examination at 8 years showed weight of 11 kg (−4.5 SD), height of 107 cm (−3.5 SD) and head circumference of 40 cm (−9.2 SD). His neurological evaluation showed increased tone and deep-tendon reflexes. Brain MRI showed very mild thinning of the corpus callosum and small cerebellar vermis (Fig.2c-c¹). Echocardiography, abdominal ultrasound, skeletal survey, and ophthalmology examination were within normal limit. CBC and immunoglobulins were within normal range. Karyotype was normal and male (46, XY).

Previously reported cases:

We identified 7 WBS patients with *DDX11* variants reported in English journals [van der Lelij et al., 2010a; Capo-Chichi et al., 2013; Bailey et al., 2015; Eppley et al., 2017]. The clinical information on these cases is summarized in Table I and the molecular detail on each of the *DDX11* causative variants is summarized in Table II. A review of the cytogenetic methods used to assess chromosome instability and sister chromatid repulsion is summarized in Table III.

Cytogenetic methods:

G- and C-banding: Conventional cytogenetic preparations were obtained from 72 hour, thymidine-synchronized, PHA-stimulated, peripheral whole blood cultures. Following treatment with Karyomax Colcemid (GIBCO) for 15 minutes, cultures were harvested according to standard cytogenetic protocol using hypotonic 0.062 M KCl and Carnoy's fixative.

Slides were made by dropping the fixed cell suspension onto precleaned slides in a Thermotron and aged for 90 minutes at 90°C. G-banding was carried out using 4× USP Pancreatin (diluted to 0.4×) followed by staining with Leishmann/Giemsa (Harleco) stain. C-banding was performed on slides pre-treated with 2% Barium Hydroxide at 60°C for 5 minutes, followed by staining in 20% Leishman Giemsa for 8-10 minutes. For each patient, 20 metaphases were analyzed for G- and C-banding studies.

Breakage studies—Chromosome preparations for breakage studies were prepared from 72 hour, peripheral whole blood cultures. Cultures were set up for each patient and a control, in triplicate with RTU alpha-MEM culture medium containing 0 (0 dose), 0.1 mcg/mL mitomycin C (MMC, Sigma), and 0.1 mcg/mL Diepoxybutane (Sigma).

For MMC cultures, following exposure to MMC for 30 minutes at 37°C, cultures were centrifuged for 10 minutes at 1000 rpm and then the supernatant aspirated. Cells were washed twice in culture medium, prior to being re-suspended in medium and returned for further incubation for 72 hours. For cultures with DEB, cultures were incubated for 24 hours and then DEB added for the latter 48 hours of the 72 hour culture period. Twenty-five minutes prior to harvesting, cultures were treated with Colcemid, exposed to hypotonic 0.062 M KCl and then fixed with Carnoy's fixative. Slides were made as described above and stained with 20% Leishman stain (Harleco) for 5-10 minutes. For each culture 50 metaphases were examined for chromosome breakage.

Slides from the '0 dose' cultures were also prepared for C-banding (as described above); for each patient, 20 metaphases were examined for centromeric morphology.

Functional analysis:

DDX11 site-directed mutagenesis —A mutation corresponding to the DDX11-R378P amino acid substitution was introduced by QuikChange site-directed mutagenesis into the 6X His-pcDNA3-3XFLAG plasmid DNA using mutagenic primers (Fp- 5' CTGCATGCGGCCACTCCGCAGGCCGCGGGCATC 3' and Rp-5' GATGCCCCGCGGCCTGCGGAGTGGCCGCATGCAG 3') and a standard protocol from Quikchange II XL site-directed mutagenesis kit (Stratagene). The mutation was confirmed by DNA sequencing.

Recombinant DDX11 protein purification —DDX11-WT and DDX11-R378P proteins were purified using a protocol previously described [Wu et al., 2012].

Modeling and secondary structure prediction —Modeling and secondary structure prediction were performed by Phyre2 server [Kelley et al., 2015].

DDX11 expression in the presence of proteasome inhibitor —3X FLAG plasmid containing DDX11-WT or DDX11-R378P cDNA were transfected in 293T cells using jetPRIME (Polyplus) transfecting reagent. After 36 h of transfection, MG132 (10 µM, Sigma) was added and the cells were incubated for an additional 14 h. Cells were then harvested and washed first with cold PBS followed by washing with cold PBS containing a protease inhibitor cocktail. Cells were lysed in RIPA buffer and approximately 30 micrograms of cell lysate protein was analyzed by SDS PAGE. The DDX11 was detected by mouse-raised FLAG antibody (1:100, Sigma) as a primary and anti-mouse HRP conjugated secondary antibody (1:1000, Invitrogen).

RESULTS

The clinical features of the 12 cases with WBS associated with *DDX11* variants are summarized in Table I. The new patients had severe prenatal and postnatal growth retardation, severe microcephaly, hearing loss and variable developmental delay, as previously described. In addition, some patients also had other structural anomalies including cardiac defects, recurrent infections, skin pigmentation and seizures.

WES revealed a compound heterozygous variant in the *DDX11* gene in patient 1 and homozygous variants in patients 2 - 5. All patients homozygous for variants were born to consanguineous parents, who were confirmed to be heterozygous carriers. Patient 3 and 4 were unrelated but had identical homozygous variant. Patient 5 had dual autosomal recessive molecular diagnosis caused by homozygous variants in *DDX11* and *MARS2* genes. The variants were confirmed via Sanger sequencing. None of the novel missense variants were located in the Fe-S cluster; however, two were located in the helicase motif V. The locations of the sequence variants including the published variants are illustrated in Fig.5A. Detailed information on each variant is listed in Table II.

Functional analysis:

Four of the five novel bi-allelic variants identified in this study can be classified as pathogenic or likely pathogenic according to ACMG guidelines [Richards et al., 2015]. Two are truncating (p.Tyr202* and p.Ala880Glyfs*94), one is supported with the cytogenetic analysis of premature centromere division (PCD) (p.Arg791Gln), and one is supported by compelling positional data as a founder variant (p.Val859Gly), see below. Only the R378P variant in patient 2 remained as a VOUS after applying the ACMG guidelines. This variant has not been seen in public databases [Clinvar, ExAC, NHLBI GO Exome Sequencing Project (ESP), gnomAD] and thus, we performed mutation analysis to clarify the effect of this variant on DDX11 function following which the variant was re-classified to “likely pathogenic”.

Novel Arg-378-Pro DDX11 variant linked to WBS —The newly discovered variant in the *DDX11* gene linked to WBS resulted in the substitution of Arginine (Arg) at position 378 with Proline (Pro). The Arg-378 resides between conserved motifs II and III of the helicase core domain found in the SF2 DNA helicases to which DDX11 belongs (Fig.5A). DDX11 also contains a conserved Fe-S cluster motif found in the sequence-related human DNA helicases Xeroderma pigmentosum (XPB), Fanconi anemia (FANCA), and

Dyskeratosis congenita (RTEL-1) [Rudolf et al., 2006; Bharti et al., 2014]. The previously reported patient variants in *DDX11*, shown in Fig.5A, did not localize to this region [van der Lelij et al., 2010a; Capo-Chichi et al., 2013; Bailey et al., 2015; Eppley et al., 2017]. Sequence comparison of the region in *DDX11* with Arg-378 revealed that it is conserved in the Fe-S helicases FANCF and RTEL-1, whereas XPD possesses an Ala at the position of Arg-378 in *DDX11* (Fig.5B).

Expression and purification of Arg-378-Pro *DDX11* —To study the impact of the Arg to Pro amino acid substitution at position 378 of *DDX11*, we introduced the desired missense variant using site directed mutagenesis and confirmed it by DNA sequencing. For biochemical studies, we attempted to purify FLAG-tagged *DDX11*-R378P recombinant proteins from 293T cells using a standard FLAG-affinity-based purification protocol [Wu et al., 2012]. The eluted proteins were resolved by SDS-PAGE. However, intact *DDX11*-R378P was hardly detectable in the eluted fraction, whereas the full-length recombinant *DDX11*-WT protein eluted from affinity FLAG-M2 resin and migrated at its expected position on SDS-PAGE, as detected by Coomassie staining of the gel (Fig.5C). Quantitation of the Coomassie-stained gels demonstrated a 5-fold less intact full-length *DDX11*-R378P mutant protein compared to wild-type *DDX11* protein ($p < 0.0001$). Western blot analyses and quantification confirmed that recombinant *DDX11*-WT was significantly more abundant [5-fold; ($p < 0.001$)] in the elution fraction compared to *DDX11*-R378P (Fig.5D). Next, we compared the level of recombinant *DDX11* proteins from total HeLa cell lysate of two different clones. By Western blot, we observed significantly less *DDX11*-R378P for either isolate compared to *DDX11*-WT (Fig.5E). By Western blot, *DDX11*-R378P was $35 \pm 18\%$ compared to *DDX11*-WT.

Modeling the structure of *DDX11* —The amino group of Proline has a cyclic ring structure, making it unfavorable to reside in an α -helical structure [Forood et al., 1993]. To assess the potential impact of the Arg-378-Pro variant on *DDX11* protein structure, we used a protein fold recognition server known as Phyre2 to model the predicted secondary structure of the regions adjacent to and containing the Arg-378 in *DDX11* with the sequence-related *Thermoplasma acidophilum* (Ta) XPD [Kuper et al., 2012]. The modeled secondary structure of *DDX11* revealed that Arg378 resides in an α -helix-forming region (Fig.6A, 6B). Moreover, this α -helix-forming region in TaXPD co-crystal structure was shown to directly interact with single-stranded DNA [Kuper et al., 2012]. Since Arg-378 in *DDX11* is likely to reside in an α -helix, it seems probable that Pro would destabilize the secondary structure. We also analyzed the effect of the Arg to Pro variant in *DDX11* at position 378 by using a program designated SuSPect (Disease-Susceptibility-based SAV Phenotype Prediction) [Yates et al., 2014] to assess the impact of the variant on *DDX11* structure and function. This program predicted a score of 62 out of 100 with a recommended cut-off of 50 for discriminating between neutral and disease-associated SAVs consistent with its destabilization of protein structure [Yates et al., 2014].

Expression of *DDX11*-Arg-378-Pro in the presence of proteasome inhibitor —The ubiquitin-proteasome pathway is responsible for degradation of misfolded cellular proteins in eukaryotic cells [Ciechanover, 1994]. We predicted that destabilized *DDX11*-

R378P might be degraded by a proteasome pathway. MG132 is a peptide aldehyde which inhibits the 26S proteasome complex from degrading proteins via the ubiquitin-proteasome pathway [Kisselev and Goldberg, 2001]. Therefore, we treated 293T cells transfected with plasmids encoding recombinant DDX11-WT or DDX11-R378P with 10 μ M MG132, and then analyzed the expression of recombinant DDX11 protein by Western blot. The level of DDX11-R378P for two different isolates was significantly increased in those transfected cells that had been treated with MG132 (Fig.6C). In the absence of the proteasome inhibitor MG132, expression of DDX11-R378P was 54 ± 2 % of DDX11-WT, whereas in the presence of MG132, DDX11-R378P was 106 ± 28 % of DDX11-WT.

Cytogenetic results:

G-band studies: Patients 1 and 2 had normal 46, XY male karyotypes in 20 metaphases examined at a G-band resolution of 550 bands.

C-band studies: This was done on patients 1 and 2. Twenty cells were examined from both the untreated (0 dose) and thymidine-synchronized cultures. For both patients, PCD was observed in all 20 metaphases from the untreated cells, with more than 80% of the metaphases showing five or more chromosomes with PCD. Metaphases from thymidine cultures also showed PCD, but the number of chromosomes with PCD was lower. In thymidine-synchronized cultures of patient 1, no PCD was observed in 6/20 cells. In the remaining 14/20 cells, chromosomes exhibiting subtle PCD ranged from 0-9 chromosomes was noted. In thymidine cultures from patient 2, all 20 metaphases showed PCD and two metaphases showed partial premature chromatid separation (PCS) (Fig.7).

Breakage studies: On patients 1 and 2, 50 metaphases were examined for chromosome breakage in three cultures (0 dose, MMC, DEB). Results were relatively consistent with the control's reference values for this laboratory and no increase in chromosome breakage was observed in any of the patient's cultures (Table III).

DISCUSSION

WBS was first reported in 2010 and named in acknowledgment of the origin of the first described patient [van der Lelij et al., 2010a]. The condition is caused by bi-allelic variants in the *DDX11* gene. DDX11 (previously called ChlR1) encodes an ATP dependent DEAD-box DNA helicase and is a member of the conserved Fe-S cluster DNA helicases [Parish et al., 2006; Hirota and Lahti, 2000]. Structurally, DDX11 contains eight conserved helicase motifs as well as a Fe-S cluster domain (Fig.5A). Functionally, DDX11 unwinds duplex DNA with a 5'-3' directionality and is essential for the correct assembly of cohesin onto DNA during mitosis [Parish et al., 2006]. WBS is classified as cohesinopathy along with Cornelia de Lange Syndrome (MIM# 122470) and Roberts Syndrome. As a group, cohesinopathies are associated with mild to severe developmental delay, intellectual disability, growth retardation, microcephaly and limb defects.

To date, there have been seven patients with WBS reported in the literature with different gene variants (Table I and II). The first case was described in 2010 by Van der Lelij et al., in a boy with severe microcephaly, pre- and postnatal growth retardation, facial dysmorphism

and abnormal skin pigmentation. Further studies demonstrated an overlapping cytogenetic phenotype between Fanconi anemia and WBS [van der Lelij et al., 2010b] and an abolishing effect of the *DDX11* mutations on helicase activity [Wu et al., 2012]. In 2012, Capo-Chichi et al. characterized a novel homozygous variant (c.788G>A) in *DDX11*, identified via homozygosity mapping and exome analysis in three Lebanese siblings presented with a severe intellectual disability and growth retardation. One of the siblings died earlier in life due to complications of tetralogy of Fallot (TOF). Subsequently, Bailey et al. [2015] described the third family with WBS in a girl with abnormal skin pigmentations and a compound heterozygous variants in the *DDX11* gene. Recently Eppley et al. [2017] reported a novel finding of small radii and fibulae in one of two siblings with WBS.

In this series, we report on five patients with novel bi-allelic *DDX11* variants identified by WES analysis. Severe microcephaly with prenatal onset was identified in all cases with no evidence of substantial decrease in the OFC over time. The patients' growth velocity was noted to be slower than the average for age and gender.

Likewise, severe pre- and postnatal growth restrictions were seen in 11/11 reported individuals. Nevertheless, two patients (patient 2 and the patient reported by Bailey et al., [2015]) had a weight between 50 to 75th centile at later life which is likely due to early initiation of G-tube feeding. One of these patients also had hypothyroidism.

To the best of our knowledge, cochlear anomalies were the main abnormality noted on temporal bone imaging in the previously reported patients. Mondini malformation was reported in one of the two siblings reported by [Eppley et al., 2017], although this term can be used indiscriminately to describe a cochleovestibular anomaly. We also noted a unique additional finding of posterior labyrinthine anomaly with persistent lateral semicircular canal anlage in patient 1 (Fig.1b-c).

As absence of the cochlear nerve renders patient ineligible for cochlear implantation, the presence of a nerve albeit small may allow consideration of cochlear implantation. Those children usually present early in life with a failed newborn hearing screen or severe speech disability comparable to the degree of sensorineural hearing loss. Expressive language was consistently affected, but other aspects of communications were also commonly involved.

Brain imaging showed left frontal focal lissencephaly, partial callosal agenesis and enlarged cisterna magna in patient 4. Microcephaly vera was seen in patients 1, 2 and 5 and mild thinning of the corpus callosum and a small cerebellar vermis in patient 5. As the cerebellar findings were unique to WBS patient cohort and since the parents were consanguineous, we looked for the possibility of another gene to explain this radiological finding. Further review of the WES data revealed a likely pathogenic homozygous variant (NM_138395.3:c.1595C>G, p.(Ala532Gly)) in *MARS2* gene (MIM# 609728), associated with cerebellar atrophy [Thiffault et al., 2006; Alfares et al., 2018]. The variant was absent from gnomAD and our internal control database but presented in his sibling who also had cerebellar atrophy on brain imaging and heterozygote for the familial *DDX11* variant. We conclude that the homozygous variant in *MARS2* gene is the cause of the cerebellar abnormalities in patient 5.

Thus, this likely represents a dual molecular diagnosis, which has been reported before especially in the setting of consanguinity [Monies et al., 2017; Yavarna et al., 2015].

Craniofacial characteristics showed a specific head shape pattern related to the microcephaly. Patient 4 had premature fusion of cranial sutures which could be secondary to poor brain development and severe microcephaly. Most patients had a depressed nasal bridge, a broad nasal tip, and overhanging columella. Other craniofacial manifestations, although commonly identified were nonspecific. Moreover, limb and skeletal anomalies were commonly affected in WBS patients, which is consistent with the clinical phenotype of other cohesinopathies. These abnormalities include fifth finger clinodactyly, brachydactyly, proximal insertion of thumbs, partial 2/3 toes syndactyly and overlapping toes. Skeletal anomalies including severe talipes equino varus and small fibula were each reported in a single case.

Additionally, we noted variable congenital cardiac anomalies in 42% (5/12) of the cases. The cardiac developmental defects were investigated earlier in *cetus*, a mouse ENU-induced mutation in *Ddx11* [Cota and García-García, 2012], which revealed abnormal heart looping and showed the role of *Ddx11* in embryonic mesoderm development and gastrulation.

The diagnosis of WBS can be supported by a combination of increased induced chromosomal breakage and sister chromatid repulsion. Elevated induced chromosomal breakage was noted in six out of eight reported patients (Table III). Review of the published information regarding chromosome breakage level in patients with *DDX11* variants showed marked elevation of the induced chromosomal breakage in the first two published reports [van der Lelij et al., 2010a; Capo-Chichi et al., 2013]. In the subsequent publications, we noted a considerable lower level of induced chromosomal breakage than what is usually seen in Fanconi anemia [Bailey et al., 2015; Eppley et al., 2017]. Studies in two of our patients did not show increased breakage. Each of the published reports had used different concentrations and exposure times to the clastogenic agents which could be the reason for the variability in the reported breakage levels (Table III). It is also possible that genotype-phenotype correlation could play a role in the variability of the results. On the other hand, cohesion defects (PCD and PCS) were consistent in most proportion of metaphases among the reported cases (Table III). We noted that evidence of PCD is more clearly discernible by C-banding studies of metaphases from untreated 72 hour cultures. There were no pathogenic variants in the known cohesinopathy genes and the coverage for these genes were between 96.56 to 100%, in our patients with cytogenetic findings of cohesion defects (Supplemental Table SI).

We conclude that unlike Fanconi anemia, elevated chromosomal breakage in WBS is variable and is not a diagnostic feature of the condition. Nevertheless, it is still a valuable tool in supporting the diagnosis and assisting variant interpretation, especially when combined with sister chromatid cohesion study.

In view of the above results, we would like to suggest renaming the syndrome to Warsaw Syndrome and would recommend the following investigation for patients with WBS: cytogenetic study of chromosome breakage and centromere cohesion, hearing assessment,

temporal bone CT-scan/MRI, developmental assessment, echocardiogram and abdominal ultrasound.

DDX11 is essential for genome maintenance and may act as a tumor suppressor [Parish et al., 2006]. As a result, the possibility of increased risk of malignancy in heterozygous carriers was raised [van der Lelij et al., 2010a]. In the previously reported patients' relatives, the mother of the patient reported by van der Lelij et al., (2010) had lymphoma and another relative, who was confirmed to be a carrier of the *DDX11* familial variant, had endometrial adenocarcinoma [van der Lelij et al., 2010a]. However, none of the affected patients and their parents had cancers and the carrier status for the *DDX11* familial variants in the relatives with malignancies was not available for analysis. Thus, the association of heterozygous, compound heterozygote and homozygous variants in the *DDX11* gene with malignancies is not clear. A larger cohort is needed to clarify this possibility.

Of note, the WES revealed a homozygous variant in *DDX11* [NM_030653.3: c.2576T>G, (p.Val859Gly)] in two apparently unrelated Saudi patients (patients 3 and 4). However, genotype analysis confirmed that both share the same haplotype. Furthermore, by querying the Saudi Human Genome Program (SGHP) database, we found 2 carriers among 3204 screened Saudi individuals, which suggests a carrier frequency of 1 in 1,602 and a disease burden of 7.52 per 1,000,000 for this founder variant (using qF instead of q2 given the inbred nature of the population where F is the population average inbreeding coefficient [Abouelhoda et al., 2016]).

Another homozygous variant (R378P) had unclear effect on *DDX11* function but was identified in patient 2 who had suggestive clinical phenotype of WBS. This novel variant occurs at a conserved position across species and has not been seen in public databases [Clinvar, ExAC, NHLBI, Exome Sequencing Project (ESP), gnomAD]. Multiple *in silico* tools predicted R378P as probably damaging to the protein structure and function. We thus performed functional analysis of the R378P variant which showed a damaging effect on *DDX11* protein stability. Thus, this variant is responsible for the WBS phenotype.

In conclusion, our series further delineates and expands the phenotype of WBS. We propose a clinical triad which include severe microcephaly, hearing loss associated with cochlear anomalies, and IUGR as the minimal criteria for WBS, especially in the context of consanguinity. Further long-term follow-up of such patients will provide valuable data for the natural history of the condition and will provide an answer to the question of increased rate of malignancies in the patients, their obligate heterozygote parents and other members of their families.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank the families for their participation in this project. The technical help of the Sequencing and Genotyping Core Facilities at KFSHRC is acknowledged. No research funding was used for this article. The characterization of the p.R378P variant was performed in the laboratory of Dr. Robert M. Brosh Jr, who is supported by the National

Institutes of Health, National Institute on Aging. We thank Joshua Sommers (NIA-NIH) for assistance in quantitation of Western blots and Coomassie-stained SDS polyacrylamide gels. We acknowledge the support of the Saudi Human Genome Program.

REFERENCE:

- Abouelhoda M, Sobahy T, El-Kalioby M, Patel N, Shamseldin H, Monies D, Al-Tassan N, Ramzan K, Imtiaz F, Shaheen R, Alkuraya FS. 2016 Clinical genomics can facilitate countrywide estimation of autosomal recessive disease burden. *Genet. Med. Off. J. Am. Coll. Med. Genet* 18: 1244–1249.
- Alfares A, Aloraini T, Subaie LA, Alissa A, Qudsi AA, Alahmad A, Mutairi FA, Alswaid A, Alothaim A, Eyaid W, Albalwi M, Alturki S, Alfadhel M. 2018 Whole-genome sequencing offers additional but limited clinical utility compared with reanalysis of whole-exome sequencing. *Genet. Med*
- Bailey C, Fryer AE, Greenslade M. 2015 Warsaw Breakage Syndrome – A further report, emphasising cutaneous findings. *Eur. J. Med. Genet* 58: 235–237.
- Bharti SK, Khan I, Banerjee T, Sommers JA, Wu Y, Brosh RM. 2014 Molecular functions and cellular roles of the ChlR1 (DDX11) helicase defective in the rare cohesinopathy Warsaw breakage syndrome. *Cell. Mol. Life Sci* 71: 2625–2639. [PubMed: 24487782]
- Capo-Chichi J-M, Bharti SK, Sommers JA, Yammine T, Chouery E, Patry L, Rouleau GA, Samuels ME, Hamdan FF, Michaud JL, Brosh RM, Jr, Mégarbane A, Kibar Z. 2013 Identification and Biochemical Characterization of a Novel Mutation in *DDX11* Causing Warsaw Breakage Syndrome. *Hum. Mutat* 34: 103–107. [PubMed: 23033317]
- Ciechanover A 1994 The ubiquitin-proteasome proteolytic pathway. *Cell* 79: 13–21. [PubMed: 7923371]
- Cota CD, García-García MJ. 2012 The ENU-induced cetus mutation reveals an essential role of the DNA helicase DDX11 for mesoderm development during early mouse embryogenesis. *Dev. Dyn* 241: 1249–1259. [PubMed: 22678773]
- Eppley S, Hopkin RJ, Mendelsohn B, Slavotinek AM. 2017 Clinical Report: Warsaw Breakage Syndrome with small radii and fibulae. *Am. J. Med. Genet. A* 173: 3075–3081. [PubMed: 28960803]
- Forood B, Feliciano EJ, Nambiar KP. 1993 Stabilization of alpha-helical structures in short peptides via end capping. *Proc. Natl. Acad. Sci. U. S. A* 90: 838–842. [PubMed: 8430094]
- Hirota Y, Lahti JM. 2000 Characterization of the enzymatic activity of hChlR1, a novel human DNA helicase. *Nucleic Acids Res* 28: 917–924. [PubMed: 10648783]
- Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJE. 2015 The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc* 10: 845–858. [PubMed: 25950237]
- Kisselev AF, Goldberg AL. 2001 Proteasome inhibitors: from research tools to drug candidates. *Chem. Biol* 8: 739–758. [PubMed: 11514224]
- Kuper J, Wolski SC, Michels G, Kisker C. 2012 Functional and structural studies of the nucleotide excision repair helicase XPD suggest a polarity for DNA translocation. *EMBO J* 31: 494–502. [PubMed: 22081108]
- van der Lelij P, Chrzanowska KH, Godthelp BC, Rooimans MA, Oostra AB, Stumm M, Zdzienicka MZ, Joenje H, de Winter JP. 2010a Warsaw Breakage Syndrome, a Cohesinopathy Associated with Mutations in the XPD Helicase Family Member DDX11/ChlR1. *Am. J. Hum. Genet* 86: 262–266. [PubMed: 20137776]
- van der Lelij P, Oostra AB, Rooimans MA, Joenje H, de Winter JP. 2010b Diagnostic Overlap between Fanconi Anemia and the Cohesinopathies: Roberts Syndrome and Warsaw Breakage Syndrome. *Anemia* 2010: 1–7.
- Monies D, Abouelhoda M, AlSayed M, Alhassnan Z, Alotaibi M, Kayyali H, Al-Owain M, Shah A, Rahbeeni Z, Al-Muhaizea MA, Alzaidan HI, Cupler E, Bohlega S, Fageih E, Faden M, Alyounes B, Jaroudi D, Goljan E, Elbardisy H, Akilan A, Albar R, Aldhalaan H, Gulab S, Chedrawi A, Al Saud BK, Kurdi W, Makhseed N, Alqasim T, El Khashab HY, Al-Mousa H, Alhashem A, Kanaan I, Algoufi T, Alsaleem K, Basha TA, Al-Murshedi F, Khan S, Al-Kindy A, Alnemer M, Al-Hajjar S, Alyamani S, Aldhekri H, Al-Mehaidib A, Arnaout R, Dabbagh O, Shagrani M, Broering D, Tulbah M, Alqassmi A, Almugbel M, AlQuaiz M, Alsaman A, Al-Thihli K, Sulaiman RA, Al-Dekhail W, Alsaegh A, Bashiri FA, Qari A, Alhomadi S, Alkuraya H, Alsebayel M, Hamad MH,

- Szonyi L, Abaalkhail F, Al-Mayouf SM, Almojalli H, Alqadi KS, Elsiey H, Shuaib TM, Seidahmed MZ, Abosoudah I, Akleh H, AlGhonaïum A, Alkharfy TM, Al Mutairi F, Eyaid W, Alshanbary A, Sheikh FR, Alsohaibani FI, Alsonbul A, Al Tala S, Balkhy S, Bassiouni R, Alenizi AS, Hussein MH, Hassan S, Khalil M, Tabarki B, Alshahwan S, Oshi A, Sabr Y, Alsaadoun S, Salih MA, Mohamed S, Sultana H, Tamim A, El-Haj M, Alshahrani S, et al. 2017 The landscape of genetic diseases in Saudi Arabia based on the first 1000 diagnostic panels and exomes. *Hum. Genet* 136: 921–939. [PubMed: 28600779]
- Parish JL, Rosa J, Wang X, Lahti JM, Doxsey SJ, Androphy EJ. 2006 The DNA helicase ChlR1 is required for sister chromatid cohesion in mammalian cells. *J. Cell Sci* 119: 4857–4865. [PubMed: 17105772]
- Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, ACMG Laboratory Quality Assurance Committee. 2015 Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med. Off. J. Am. Coll. Med. Genet* 17: 405–424.
- Rudolf J, Makrantonis V, Ingledew WJ, Stark MJR, White MF. 2006 The DNA Repair Helicases XPD and FancJ Have Essential Iron-Sulfur Domains. *Mol. Cell* 23: 801–808. [PubMed: 16973432]
- Thiffault I, Rioux MF, Tetreault M, Jarry J, Loiselle L, Poirier J, Gros-Louis F, Mathieu J, Vanasse M, Rouleau GA, Bouchard JP, Lesage J, Brais B. 2006 A new autosomal recessive spastic ataxia associated with frequent white matter changes maps to 2q33–34. *Brain* 129: 2332–2340. [PubMed: 16672289]
- Wu Y, Sommers JA, Khan I, de Winter JP, Brosh RM. 2012 Biochemical Characterization of Warsaw Breakage Syndrome Helicase. *J. Biol. Chem* 287: 1007–1021. [PubMed: 22102414]
- Wu Y, Suhasini AN, Brosh RM. 2009 Welcome the Family of FANCDJ-like Helicases to the Block of Genome Stability Maintenance Proteins. *Cell. Mol. Life Sci. CMLS* 66: 1209–1222. [PubMed: 19099189]
- Yates CM, Filippis I, Kelley LA, Sternberg MJE. 2014 SuSPect: enhanced prediction of single amino acid variant (SAV) phenotype using network features. *J. Mol. Biol* 426: 2692–2701. [PubMed: 24810707]
- Yavarna T, Al-Dewik N, Al-Mureikhi M, Ali R, Al-Mesaifri F, Mahmoud L, Shahbeck N, Lakhani S, AlMulla M, Nawaz Z, Vitazka P, Alkuraya FS, Ben-Omran T. 2015 High diagnostic yield of clinical exome sequencing in Middle Eastern patients with Mendelian disorders. *Hum. Genet* 134: 967–980. [PubMed: 26077850]

Fig. 1: CT and MRI of the inner ear in order of the degree of cochlear hypoplasia. Patient 1 (a-d), patient 3 (e-h), patient 2 (i-l) and patient 4 (m-p).

Axial CT image (a) demonstrates lateral tapering (arrow) of the basal turn at the junction of the ductus reuniens. Axial CT (b) and axial T2W (c) images demonstrate a cystic cochlea (arrows) and a persistent anlage of the lateral semicircular canal (open arrows). Sagittal T2W image (d) reveals 4 nerves in the internal auditory canal. The cochlear nerve (arrow) is present, but smaller than the other nerves.

Axial CT (e) demonstrates a hypoplastic basal turn and cochlea (arrow). Axial CT (f) demonstrates an amorphous cystic cochlea (arrow) and a hypoplastic cochlear nerve canal

(black arrow). Axial CT image (g) demonstrates a persistent anlage of the posterior semicircular canal (open arrow), lacking a bone island. Axial CT (h) reveals a truncated superior semicircular canal with only the anterior limb (arrow). The apex and posterior limb are absent.

Axial CT image (i) reveals a tiny cochlea (arrow). Axial CT image (j) reveals a normal bone island within the lateral semicircular canal (open arrow). Axial T2W (k) image shows a tiny cochlea (arrow). Sagittal T2W image (l) reveals only 2 nerves (facial and vestibular nerves) (arrow) in the internal auditory canal. The cochlear nerve is absent and the internal auditory canal is small.

Axial CT (m) reveals absent basal turn of the cochlea (*), while axial CT (n) demonstrates a hypoplastic cochlea (black arrow). 3D surface reconstruction (o) also confirms cochlear hypoplasia (arrow) and absent basal turn, while lack of cochlear nerve (white arrow) is shown on axial T2W MRI (l). The semicircular canals and vestibule are normal.

Fig. 2: MRI findings on patient 1, 2 and 5.

Patient 1 (a/a¹), Patient 2 (b/b¹) MRI images obtained at 8 months of age. Patient 5 (c/c¹) MRI obtained at 15 months of age. All 3 patients show similar microcephaly with simplified gyri and age appropriate myelin maturation. There is metric breaking in all, consistent with early suture closure. Sagittal T1W image (a) of patient 1 demonstrates a normal corpus callosum, brainstem and vermis. Axial T2W image (a¹) shows normal ventricular size. Sagittal T2W image (b) in patient 2 shows similar findings. Axial T2W image (b¹) in patient 2 shows mild ventricular and pericerebral CSF prominence (correlate with HC in each). Sagittal T1W image (c) in patient 5 reveals very mild thinning of the corpus callosum, small cerebellar vermis and mildly prominent 4th ventricle. Axial T1W image (c¹) in patient 5 demonstrates normal lateral and third ventricular size and pericerebral CSF spaces.

Fig. 3: Facial features of individuals with WBS.

(A1-2) patient 1 at 2 years of age showing flat supraorbital ridges, depressed nasal bridge, broad nasal tip, overhanging columella, normal philtrum, prominent upper lip, retrognathia and small cupped ears.; (B1-2) patient 2 at 2 years of age with a short forehead, triangular and small face, arched eyebrows, deep-set eyes, flat nasal bridge, short prominent nose with overhanging columella, short philtrum, full lips and retrognathia.; (C1-2) patient 3 at 6 years of age. Note short forehead, small face, deep-set eyes, epicanthic folds, droopy eyelids, depressed nasal bridge, overhanging columella, short philtrum, full lips, and retrognathia.; (D1-2) patient 5 at 5 years of age (D1) and at 6 years of age (D2) with a triangular small face, receding short forehead, prominent eyes, beaked nose, narrow nostrils, overhanging columella, short philtrum, thin lips and retrognathia, posteriorly rotated ears with marked prominence of antihelix and small and attached earlobes with prominent tragus.

Fig.4: Lower limbs of individuals with WBS.

(A) patient 2 at 2 years of age with pes planus, clinodactyly of the left 5th toe. The 5th toe overlaps the 4th toe, bilaterally. On the right, the 4th toe overlaps the 3rd toe. The 5th toe was proximally inserted, and there was 2/3 toes partial syndactyly, bilaterally. (B) patient 3 at 6 years of age with bilateral clinodactyly of the 5th toe and hypoplastic toe nails. On the right, the left 5th and 4th toes were proximally inserted and there was 2/3 toes partial syndactyly. (C) patient 5 at 5 years of age with bilateral clinodactyly of the 3rd, 4th, and 5th toes and the 2nd toe overlaps the 3rd toe.

Fig. 5: WBS-linked Arg-378-Pro variant in DDX11.

(A) Location of disease-linked DDX11-R378P variant with respect to conserved helicase core motifs and Fe-S cluster. Conserved helicase motifs are shown in red and Fe-S motif is highlighted in yellow. Previously published variants in *DDX11* shown in black and the novel variants from this series shown in blue. (B) Sequence alignment (Muscle Multiple Sequence Alignment) of region spanning R378 residue of DDX11 and related Fe-S containing DNA helicases XPD, FANCI, and RTEL-1. (C) Coomassie-stained SDS-PAGE gel to analyze FLAG-M2 resin affinity-purified FLAG-tagged DDX11-WT and DDX11-R378P

recombinant proteins expressed in 293T cells. E-elution. The intact full-length DDX11-R378P protein was hardly detectable in the eluted fraction compared to the full-length DDX11-WT protein. (D) Western blot analysis and quantification of DDX11 protein samples. Panel A. Sup- the supernatant obtained after centrifugation of cell lysates; Elution- Proteins obtained after 3X FLAG peptide elution in the final stage of purification. The quantification shows ~5-fold greater in wild-type DDX11 protein compared to the R378P DDX11 protein. (E) Western blot analysis of whole cell lysate protein from HeLa cells expressing recombinant DDX11-WT or DDX11-R378P. Actin serves as a loading control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig.6: Predicted and apparent destabilization of DDX11 protein by R378P mutation.

(A) Alignment of secondary structure of DDX11 based on *Thermoplasma acidophilum* (Ta) XPD as a template using Phyre2 protein fold recognition server. Region of secondary structure spanning R378 amino acid (in Block) is shown. (B) Predicted modeled structure of DDX11 protein. Human DDX11 protein was modeled by Phyre2 protein fold recognition server. Structure is based on homology modeling using TaXPD (Kuper et al., 2012). Arg-378 with side chain (ball and stick) is shown and indicated in edged red surface. (C) Effect of Proteasome inhibitor MG132 on the expression of DDX11 proteins. 293T cells transfected with DDX11-WT, DDX11-R378P#1 (R378P#1), or DDX11-R378P#2 (R378P#2) were either treated with MG132 (10 μ M) or DMSO used as a not treated (NT) for 14 h. Cell lysates were prepared and resolved by SDS-PAGE, followed by Western blot detection with FLAG antibody. Actin was used as a loading control.

Fig.7: C-banding of metaphase chromosomes in patients 1 and 2. Short arrows show chromosome morphology suggestive of PCD; long arrows show chromosomes with PCS
(A) Representative metaphase from untreated (0 dose) culture of patient 1; (B) Thymidine-synchronized culture of patient 2.

TABLE I:

Clinical phenotype reported in patients with WBS.

	Van der Lelij et al., 2010 (N=1)	Capo-Chichi et al., 2013 (N=3 siblings)	Bailey et al., 2015 (N=1)	Eppley et al., 2017 (N=2 siblings)	Patient (1)	Patient (2)	Patient (3)	Patient (4)	Patient (5)	Total N= 12
Sex	M	(F:2) ; (M:1)	F	(F:2)	M	M	F	M	M	(F:6) ; (M:6)
Ethnicity	Polish	Lebanese	British	European/ Native American	Croatian / Italian	Pakistani	Saudi	Saudi	Egyptian	
Consanguinity	-	+	-	-	-	+	+	+	+	7/12
Prenatal IUGR	+	2/2	+	2/2	+	+	+	+	+	11/11
Postnatal Growth Restriction	+	2/2	+	2/2	+	+	+	+	+	11/11
Microcephaly	+	2/2	+	2/2	+	+	+	+	+	11/11
SNHL	+	3/3	+	2/2	+	+	+	+	+	12/12
Cochlear Hypoplasia or Functional Abnormalities	+	2/2	+	2/2	+	+	+	+	NA	10/10
Imaging										
Brain Structural Abnormalities	NA	NA	NA	NA	+	+	NA	+	+	4/4
Developmental Delay										
Language	+	NA	+	2/2	+	+	+	+	+	9/9
Intellectual Disability	+	3/3	+	2/2	+	+	+	+	+	12/12
Gross and Fine Motor	+	0/1	NA	0/2	-	+	-	-	+	3/9
Hypotonia	NA	1/1	NA	2/2	-	+	-	-	-	4/8
Dysmorphism:										
Head and Face	+	3/3	+	2/2	+	+	+	+	+	12/12
Ears	+	NA	+	2/2	+	+	+	NA	+	8/8
Nose	NA	3/3	+	2/2	+	+	+	+	+	11/11
Eye	+	1/2	+	2/2	+	+	+	+	+	10/11
Mouth/ Philtrum	+	1/2	+	NA	+	+	+	NA	+	7/8
Skeletal										
Fingers, Toes	+	3/3	+	2/2	+	+	+	+	+	12/12

	Van der Lelij et al., 2010 (N=1)	Capo-Chichi et al., 2013 (N=3 siblings)	Bailey et al., 2015 (N=1)	Eppley et al., 2017 (N=2 siblings)	Patient (1)	Patient (2)	Patient (3)	Patient (4)	Patient (5)	Total N= 12
Other Skeletal	NA	NA	NA	Small radii and fibulae (1/1)	-	-	Talipes equino varus	Craniosynostosis	-	3/6
Cardiac	VSD	TOF (1/3)	PDA	0/2	-	Large PDA, small ASD	VSD	-	-	5/12
Renal/ Multicystic Kidney	NA	0/2	+	NA	NA	-	NA	-	-	1/6
Genitalia	-	NA	NA	NA	-	+	NA	NA	+	2/4
Recurrent Infections	-	NA	-	2/2	-	-	+	-	+	4/9
Skin Pigmentation	+	0/3	+	2/2	-	+	+	-	-	6/12
Family History of Malignancy	+	NA	NA	NA	+	+	-	NA	-	3/5

N- number; NA - Not available; M- male; F- female; IUGR - Intrauterine growth restriction; SNHL - Sensorineural hearing loss

TABLE II:

DDX11 gene variants currently reported.

	Ref Seq ID	Exon	Codon	Protein	Predicted effect	Domain	SIFT [‡]	Mutation Taster [‡]	Mutation Assessor [§]	PolyPhen-2 [¶]	CADD_Phred [✕] %
Patient 1	NM_030653.3	Exon 5	c.606delC	p.Tyr202*	frameshift	Helicase core	NA	Disease causing	NA	NA	NA
Patient 1	NM_030653.3	Exon 23	c.2372G>A	p.Arg791Gln	missense	Helicase motif V	Deleterious (0.02)	Disease causing	High	Probably damaging (0.937)	33
Patient 2	NM_030653.3	Exon 10	c.1133G>C	p.Arg378Pro	missense	Helicase core	Deleterious (0)	Disease causing	High	Probably damaging (0.997)	25.1
Patient 3 and 4	NM_030653.3	Exon 26	c.2576T>G	p.Val859Gly	missense	Helicase motif V	Deleterious (0.01)	Disease causing	Medium	Benign (0)	22.5
Patient 5	NM_030653.3	Exon 26	c.2638dupG	p.Ala880Glyfs*94	frameshift	Helicase motif V	NA	Disease causing	NA	NA	NA
Van der Lelij et al., 2010	NM_030653.3	Intron 22	c.2271+2T>C (IVS22+2T>C)	p.Cys754Profs*9	frameshift	Helicase core	NA	Disease causing	NA	NA	21.7
Van der Lelij et al., 2010	NM_030653.3	Exon 26	c.2689_2691del	p.Lys897del	in frame deletion	C-terminal	NA	Disease causing	NA	NA	NA
Capo-Chichi et al., 2013	NM_030653.3	Exon 7	c.788G>A	p.Arg263Gln	missense	Fe-S cluster	Deleterious (0)	Disease causing	High	Probably damaging (1)	28.4
Bailey et al., 2015	NM_030653.3	Intron 5	c.638+1G>A	Splice site		Helicase core	NA	Disease causing	NA	NA	22.7
Bailey et al., 2015	NM_030653.3	Exon 19	c.1888delC	p.Arg630Glyfs*23	frameshift	Helicase core	NA	Disease causing	NA	NA	NA
Eppley et al., 2017	NM_030653.3	Intron 19	c.1949-1G>A (IVS19-1G>A)	Splice site		Helicase core	NA	Disease causing	NA	NA	25
Eppley et al., 2017	NM_030653.3	Exon 16	c.1523T>G	p.Leu508Arg	missense	Helicase core	Deleterious (0)	Disease causing	Medium	Probably damaging (0.999)	24.3

[‡]<http://sift.jcvi.org>

[‡]<http://www.mutationtaster.org>

[§]<http://mutationassessor.org/>

[¶]<http://genetics.bwh.harvard.edu/pph2/>

[✕]<http://cadd.gs.washington.edu/>

[%] CADD scoring: 10 indicates the top 10% most deleterious substitutions in the human genome, 20 indicates the top 1% most deleterious, 30 indicates the top 0.1% most deleterious.

TABLE III:

Results of the cytogenetic analysis and chromosome breakage in the reported patients with WBS.

	Van der Lelij et al., 2010a (N=1)	Van der Lelij et al., 2010b (N=1)	Capo-Chichi et al., 2013 (N=3 siblings)	Bailey et al., 2015 (N=1)	Eppley et al., 2017 (N=2 siblings)	This study (Patients 1 and 2)
Tissue	Lymphocytes	Lymphocytes and fibroblasts	Lymphocytes	Lymphocytes	Lymphocytes	Lymphocytes
Clastogenic Agent	MMC	MMC	MMC	DEB	DEB	MMC and DEB
Final Concentration in Culture	150nM (0.05 µg/mL)	300nM (0.1 µg/mL)	9nM (0.003µg/mL)	NA	0.1 µg /mL	0.1 µg /mL
Exposure Time	48 hr	72 hr	24 hr	NA	NA	72 hr (MMC) 48 hr (DEB)
Elevated Induced Chromosome Break	+	+	+	+	+	-
Elevated Spontaneous Chromosome Break	-	-	-	-	-	-
Metaphases with PCS	50-60%	79-98%	Patient 1: 46.5% Patient 3: 44% (2/2)	+	NA	Patient 1: 100% Patient 2: 100% (2/2)