A novel splice-site mutation of *WRN* (c.IVS28+2T>C) identified in a consanguineous family with Werner Syndrome

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Abstract. Werner Syndrome (WS) is a rare, adult-onset progeroid syndrome that is associated with multiple age-associated complications and relatively short life expectancy. The characteristics of WS include a 'bird-like' appearance, canities, cataracts and ulcerations around the ankles. In addition, certain patients develop hypogonadism with atrophic genitalia and infertility. The average life span of affected individuals is 54 years. Previous studies have demonstrated that mutations in the Werner syndrome RecQ like helicase gene (*WRN*) may contribute to WS. The present study investigated a consanguineous family with WS, comprising of 4 generations from Northwest China (Gansu province). A novel homozygous splice-site mutation in *WRN* (c.IVS28+2T>C) was identified in this family and was predicted to be deleterious. No further relevant mutations were identified by direct sequencing of the genes lamin A/C, barrier to autointegration factor 1, zinc metallopeptidase STE24 and DNA polymerase Δ1. cDNA sequencing and alignments were performed to further confirm the pathogenicity of this mutation. The results support the important role of *WRN* in WS and expand the spectrum of known *WRN* mutations. In addition, it may provide novel approaches in genetic diagnosis and counseling of families with WS.

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Introduction

Werner Syndrome (WS; OMIM entry no. 277700; https://omim.org/entry/277700) is a rare progeroid syndrome associated with a number of aging phenotypes. WS often occurs in consanguineous families and affects ~1 in 1 million individuals in the general population (1-3). Patients with WS often exhibit adult-onset progeria and have an increased risk of developing cancer (4). At birth, patients with WS do not present any clinical symptoms; the lack of pubertal growth spurts are generally the first symptom identified (5). Over time, the typical characteristics of WS become evident, including a 'bird-like' appearance, cataracts, canities, ulcerations around the ankles and certain patients develop hypogonadism with atrophic genitalia and infertility (6). The average life span of affected individuals is 54 years (5).

WS is caused by mutations in the Werner syndrome RecQ like helicase gene (*WRN*), which was first cloned in 1996 (7). *WRN* is located on chromosome 8p11-p12, spanning ~250 kb and consists of 35 exons, 34 of which are protein coding (7). *WRN* is generally considered to follow an autosomal-recessive pattern of inheritance (8). *WRN*, coding a 180 kDa multifunctional nuclear protein, belongs to the family of RecQ type helicases (9). Sequence analysis and subsequent biochemical analysis have revealed that human WRN possesses helicase and exonuclease functions (10). It serves a role in DNA replication, transcription, repair, recombination and heterochromatin maintenance (including telomere maintenance), indicating that one of the major causes of WS pathogenesis may be associated with genomic instability (11). In the absence of functioning WRN, cells accumulate potentially toxic DNA intermediates or critically short telomeres, which induce genetic instability, misexpression and mutagenesis (12-14). In addition, they may drive cell loss and produce tissue-specific defects (15). Compromised cell or tissue structure and function leads to two seemingly divergent outcomes: Senescence and neoplasms (16,17). To date, the majority of disease-inducing mutations in *WRN* are truncating mutations (5).

In addition to *WRN*, a small number of heterozygous mutations in the lamin A/C gene (*LMNA*) have been revealed to produce similar phenotypes to WS, which suggests that *LMNA* may be an underlying disease-inducing gene in

WS (3,18,19). *LMNA* encodes nuclear intermediate filaments, lamin A and lamin C (1). A previous study demonstrated that WS patients with *LMNA* mutations exhibited a younger onset when compared to patients with classical WS (5). In addition, a number of studies have suggested that barrier to autointegration factor 1 (*BANF1*), zinc metallopeptidase STE24 (*ZMPSTE24*) and DNA polymerase Δ1 (*POLD1*) may be involved in progeroid syndrome (15,20,21).

The present study investigated the potential causative gene in a consanguineous family with WS from Northwest China. A novel homozygous splice-site mutation (c.IVS28+2T>C) was identified in intron 28 of *WRN* in the proband and co-segregated with the affected WS family members. To the best of our knowledge, this mutation has not been reported in previous studies, nor was it identified in our previous control cohorts or the single nucleotide polymorphism (dbSNP) database (https://www.ncbi.nlm.nih.gov/projects/SNP/) and the Exome Variant Server database (http://evs.gs.washington.edu/EVS/).

Materials and methods

Patients. A consanguineous family from Northwest China (Gansu province) consisting of 11 living members across four generations participated in the present study (Fig. 1A). The proband, family member 2 from the 4th generation (IV:2), was diagnosed with WS. The remaining 10 members (II:2, II:4, III:1, III:2, III:3, III:4, IV:1, IV:3, IV:4 and V1) were phenotypically normal. The proband was admitted to Xiangya Hospital in April 2015 (Changsha, China) for treatment of an ankle ulcer. The 38-year-old patient (gender, male) had a 'bird-like' face, gray hair, a husky voice and a recurrent ulceration around his ankle which first presented 12 years previously (Fig. 1B). The Review Board of Xiangya Hospital of the Central South University (Hunan, China) approved this research and all family members involved gave written informed consent.

DNA extraction. Genomic DNA was extracted from the peripheral blood of the patient and the other family members using a DNeasy Blood & Tissue kit (Qiagen, Inc., Valencia, CA, USA) on the QIAcube automated DNA extraction robot (Qiagen, Inc.).

Mutation sequencing. The entire coding regions, including the flanking intronic sequences of *WRN* [Refseq (https://www.ncbi.nlm.nih.gov/refseq/), NM_000553], *LMNA* (NM_170,707), *BANF1* (NM_003860), *ZMPSTE24* (NM_005857) and *POLD1* (NM_001256849) were amplified by polymerase chain reaction (PCR; primer sequences are available upon request). PCR product sequences were determined using the ABI 3100 Genetic Analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (22).

Multiple sequence alignments and bioinformatic prediction of mutation. The multiple WRN protein sequences across mammals were aligned using the multiple sequence comparison by log-expectation program (version 3.6; https://www.ncbi.nlm.nih.gov, and the MUSCLE software) (23,24).

RNA extraction and reverse transcription for verification. Total RNA was extracted from mononuclear cells from the peripheral blood of the patient using the Nuclearspin RNA II kit (Macherey-Nagel GmbH, Düren, Germany) and DNase (DNase I, RNase-free (1 U/μl); Thermo Fisher Scientific, Inc.) treated (25). Reverse transcription (RT)-qPCR was performed to convert extracted total RNA into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions (26). cDNA was then sequenced following amplification by PCR. The PCR was conducted in a 25 μl reaction mixture, which consisted of 0.3 mM deoxyribonucleotide triphosphates, 1X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, and 0.01% w/v gelatin), 2.0 mM MgCl2, 0.5 μM of each primer (forward and reverse), 1.5 U of Taq polymerase, and 50 ng genomic DNA. The thermal cycling consisted of an initial denaturation at 95˚C for 4 min, followed by 35 cycles of amplification consisting of denaturation at 95˚C for 1 min, primer annealing at 55-61˚C for 30 sec and primer extension at 72˚C for 1 min. A final extension step was performed at 72˚C for 7 min. The results were compared to the normal control for variant analysis via multiple sequence alignment using the MUSCLE software (24).

Results

The present study investigated the case of a patient with WS (IV:2) born to consanguineous parents (family members III:2 and III:3) in a family comprised of four generations with 11 living members. The proband, with a 'bird-like' face, husky voice, canities and ankle ulcers, conforms to the typical phenotypes associated with WS. The present study investigated the potential causative genes among all family members. Sequence analysis of *WRN*, *LMNA*, *BANF1*, *ZMPSTE24* and *POLD1*, identified a previously unreported homozygous splice-site mutation in intron 28 (c.IVS28+2T>C) of the *WRN* gene in the proband (IV:2) and co-segregated with the affected family members (Fig. 1C). No further relevant mutations were identified by direct sequencing of the genes for *LMNA*, *BANF1*, *ZMPSTE24* and *POLD1*. In addition, the cDNA sequencing results from the proband identified deletion of *WRN* exon 28 (74 nucleotides; Fig. 1D). This newly discovered c.IVS28+2T>C mutation was not identified in the 200 control cohorts that our group studied previously (23). In addition, this mutation was not present in the dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/) or Exome Variant Server databases (http://evs.gs.washington.edu/EVS).

Discussion

The *WRN* gene encodes a nuclear protein which belongs to the family of RecQ type helicases and comprises of five functional domains including an exonuclease region, a helicase region, a RecQ C-terminus consensus region, an RNase D consensus region and a nuclear localization signal region (NLS) (27). According to previous studies, homology-dependent recombination repair (HDR) may be used to repair DNA damage while suppressing gene loss or rearrangement. In addition, WRN appears to serve a role late in the HDR process when recombinant molecules are topologically disentangled for segregation to daughter cells (28,29), as well as in the maintenance of telomere length and the suppression

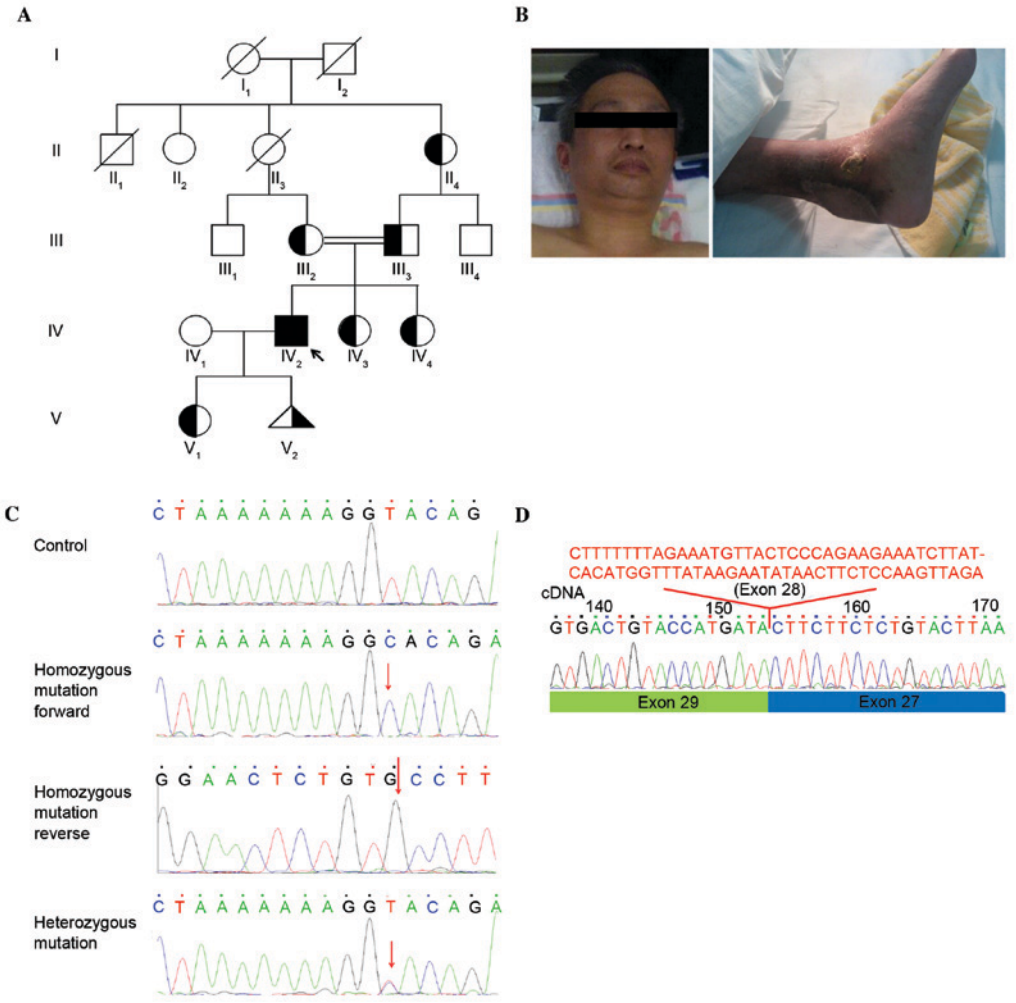

Figure 1. (A) Ancestry of the family affected with WS. Family members are identified by their generation (indicated by roman numerals) and a number. Squares represent male family members and circles, female members; the triangle represents a fetus. The black symbol represents a member with WS, the white symbols represent unaffected members and the half black-half white symbols represent carriers. The arrow indicates the proband. (B) Phenotypes of the proband. The proband has a 'bird-like' face, canities, a husky voice and a recurrent ulceration around the ankle. (C) Sequencing results of the *WRN* mutation. Sequence chromatograms indicate a homozygous splice-site mutation (c.IVS28+2T>C) in the proband. (D) The reverse sequencing results of the cDNA with the *WRN* mutation. The sequence chromatogram reveals a deletion of exon 28 following a homozygous splice-site mutation (c.IVS28+2T>C). The green rectangular box represents exon 29 and the corresponding sequence and the blue rectangular box represents exon 27 and corresponding sequence. The red letters indicate deleted nucleotides in exon 28 and the red 'Y' shape indicates the normal location of exon 28. WS, Werner syndrome; *WRN*, Werner syndrome RecQ like helicase.

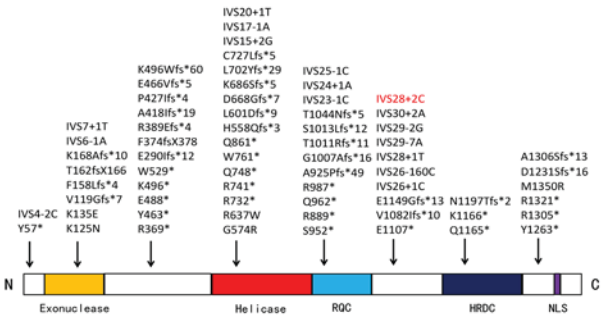

Figure 2. *WRN* mutations identified in Werner syndrome patients. The rectangular box represents the WRN protein with the N-terminus on the left and C-terminus on the right. Known functional domains include an exonuclease region, a helicase region, a RQC region, a HRDC region and a NLS. Mutations are grouped based on the mutation site. The red words represent the previous splice-site mutation. *Indicates a termination codon. *WRN*, Werner syndrome RecQ like helicase; RQC, RecQ C-terminus consensus region; HRDC, RNase D consensus region; NLS, nuclear localization signal.

of telomere sister-chromatid exchanges (30,31). WRN may also be involved in non-homologous DNA-end joining, base-excision repair, DNA-damage signaling and transcription (15). Zhang *et al* (11) revealed that the progressive heterochromatin disorganization observed in WRN-deficient mesenchymal stem cells underlies cellular aging (32). Thus, loss of WRN may disrupt genetic stability, lead to cell aging and death. Therefore, it may be pivotal in human aging and the development of WS.

The present study revealed that the novel mutation (c.IVS28+2T>C) causes a change in the splicing pattern, leading to a deletion of 74 nucleotides in the mRNA. It suggested that the substitution and subsequent skipping of exon 28 may produce a frameshift transcript, resulting in the absence of the NLS domain. The NLS domain is essential for WRN protein targeting to the nucleus via the nuclear pore complex (33). In addition, the majority of previously reported

causative WRN mutations also give rise to a lack of NLS at the C-terminus of the protein (Fig. 2) (5,34).

During this research, the wife of the proband (IV:1) was pregnant. It was speculated that the infant (V:2) may be a carrier without any pathological phenotype. However, amniocentesis or shotgun sequencing of maternal plasma DNA is required to produce accurate and convincing results to verify this. As cancer predisposition is a key feature in WS, the proband may have a higher risk of cancer development and should therefore have regular health examinations.

In conclusion, the present study identified a novel homozygous splice-site mutation (c.IVS28+2T>C) in a four generation family with WS. The present identification of a novel mutation expands the spectrum of known WRN mutations (only 87 mutations were identified as of February 2015; http://www.hgmd.cf.ac.uk/ac/search.php) and it may contribute to novel approaches to genetic diagnosis and counseling of families with WS.

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