Human Molecular Genetics, 1996, Vol. 5, No. 12 **1909–1913**

Homozygous and compound heterozygous mutations at the Werner syndrome locus

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Received June 22, 1996; Revised and Accepted September 23, 1996

The Werner syndrome (WS) is a rare autosomal recessive progeroid disorder. The Werner syndrome gene (*WRN*) has recently been identified as a member of the helicase family. Four distinct mutations were previously reported in three Japanese and one Syrian WS pedigrees. The latter mutation was originally described as a 4 bp deletion spanning a spliced junction. It is now shown that this mutation results in a 4 bp deletion at the beginning of an exon. Nine new *WRN* mutations in 10 additional WS patients, both Japanese and Caucasian, are described. These include three compound heterozygotes (one Japanese and two Caucasian). The new mutations are located all across the coding region.

INTRODUCTION

Werner syndrome (WS) is a rare autosomal recessive segmental progeroid syndrome (2). Patients exhibit not only an appearance of accelerated aging (premature graying, thinning of hair, skin atrophy and atrophy of subcutaneous fat), but also several disorders commonly associated with aging. These include bilateral cataracts, diabetes mellitus, osteoporosis, several forms of arteriosclerosis and a variety of benign and malignant neoplasms (3,4).

WS fibroblasts have very limited proliferative capacities as compared with age-matched controls (5–7). A prolongation of the S phase has been demonstrated both in WS fibroblasts and lymphoblastoid cell lines (8). Cultured cells exhibit a propensity for chromosomal and intragenic mutations (9–12). The rate of repair of X-ray- or UV-damaged DNA appears to be normal in WS fibroblasts (13).

WRN was initially mapped to chromosome 8p (14,15). Physical and genetic maps of the region were constructed (16–19). *WRN* has recently been identified (GenBank accession number L76937) and four distinct *WRN* mutations were described (1). The *WRN* gene encodes a 1432 amino acid protein partially homologous to RecQ helicases (20). The WRN protein contains seven helicase motifs; two of them have been identified in all ATP-binding proteins (21).

DNA helicases have been implicated in a number of molecular processes. One of the most important functions of DNA helicases is the unwinding of DNA during DNA replication as a component in a replication complex (22–24). Another function of helicase involves DNA repair. It has been hypothesized that some forms of nucleotide excision repair are coupled with transcription; mutant helicases responsible for the DNA instability syndromes may impair lesion recognition and/or lesion removal of the damaged nucleotides during transcription (25–27). Examples include: *ERCC2* helicase, which complements xeroderma pigmentosum B and its yeast homologue *RAD3* (28,29); *ERCC3*, which complements xeroderma pigmentosum D and its yeast homologue *RAD25* (30–34); *ERCC3* and *ERCC6*, which complement a Cockayne syndrome mutation (35,36). In *Escherichia coli*, the *RecQ* helicase is involved in the initial step of DNA repair by recombination (37).

Helicases are required for accurate chromosomal segregation. In yeast, precise chromosome segregation requires *Sgs*1, a eukaryotic homologue of *RecQ* (38).

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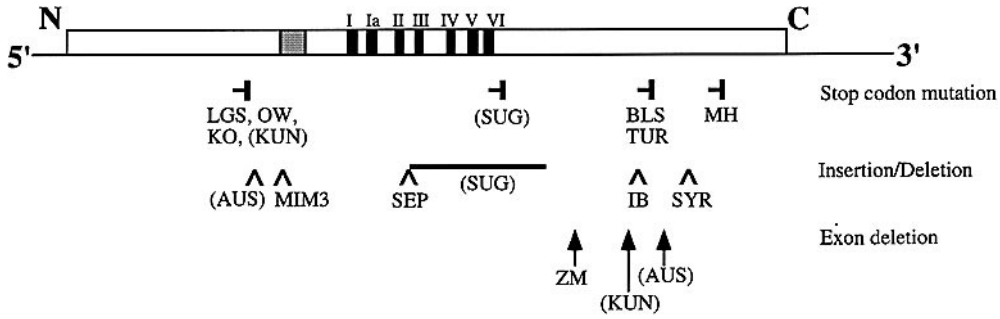

Figure 1. Locations of the *WRN* mutations. The rectangular box indicates the predicted WRN protein. The light shadowed segment indicates the highly acidic repeat region; dark shadows indicate the locations of the helicase consensus motifs. The locations of the *WRN* mutations are grouped based upon the type of mutation and are shown underneath the WRN protein along with Registry codes. Parentheses indicate the heterozygous mutations. ZM and MH mutations were previously described (1).

Given the several potential roles of the WRN protein, a careful delineation of spontaneous mutations at this locus could facilitate the characterizations of its functions. We report nine *WRN* gene mutations from 10 WS patients, three of which were compound heterozygotes. Two other mutations found in three patients have been previously reported (1). These various mutations involve sequences throughout the coding region.

RESULTS

Four Japanese and eight non-Japanese WS patients were selected from our International Registry. Six of them (AUS, KO, MIM3, SEP, TUR, UH) were classified as 'definite WS' and three (LGS, OW, SUG) as 'probable WS'. Clinical and laboratory data for members of BLS, KUN and SYR remain incomplete, but the affected subjects had been diagnosed as WS by the submitting physicians.

Three new mutations were found in regions N-terminal with respect to the helicase consensus motifs. The point mutation at nt 1336, CGA (Arg) to TGA (Stp), was found as a homozygous mutation in one Caucasian (LGS) and two consanguineous Japanese (OW, KO) WS subjects and as a heterozygous mutation in one Japanese WS subject (KUN). LGS denied consanguinity; non-consanguinity was supported by haplotype data (19). A single nucleotide deletion at 1194–1196, AAA to AA, was seen as a heterozygous mutation in AUS. This mutation would create a frameshift which ends at 1406–1408 TGA (Stp). A four nucleotide insertion (ATCT) between 1509 and 1520 was homozygous in MIM3. This frameshift mutation would terminate at 1535–1537 TGA (Stp).

Three mutations were found within or just 3′ to the helicase motifs in two Caucasian patients. One (SEP) mutation was a 105 bp insertion between 2319 and 2320. The insertion results in a termination codon, creating a truncated protein that excludes helicase domains III and the subsequent C terminus of the WRN protein. A second mutation was a deletion of nucleotide 2320–3056 seen in SUG as a heterozygous mutation, terminating at nt 3081–3083 TGA (Stp). The third mutation was a heterozygous termination mutation found in SUG, located 30 amino acids after the last helicase motif.

Three new mutations were found in regions C-terminal to the helicase motifs. A Japanese patient, IB, was homozygous for an A deletion at nt 3677. The mutated protein stops at nt 3713–3715 TAG (Stp). BLS (French) and TUR (Turkish) patients shared the same mutation at nt 3724, CGA (Gln) to TGA (Stp), which was previously found in the Japanese SY family (1). A 74 bp deletion of nt 3541–3614 was seen as a heterozygous mutation in a Japanese WS, KUN. This deletion results in a termination at 3720–3722 TAG (Stp). A 113 bp deletion of nt 3691–3803, which would result in a termination at nt 3816–3818 TGA (Stp), was found as a heterozygous deletion in the Caucasian WS, AUS.

These mutations were confirmed by sequencing of genomic PCR products, using the primers from the intron sequences of *WRN* (39). A summary of the newly discovered mutations is given in Figure 1.

The mutation in the SYR pedigree was previously reported as a 4 bp deletion at the intron–exon boundary, 2 bp from the putative intron and 2 bp from the contiguous exon (gtagACAGACC at the DNA level). This was expected to cause an in-frame deletion of the exon. Our RT–PCR protocol, however, showed a deletion of 4 bp, ACAG, from the beginning of this exon. The ACAG deletion would result in a termination at nt 3971–3973 TAG (Stp).

DISCUSSION

In our original report of the positional cloning of the *WRN* locus, four distinct homozygous mutations in the 3′ region of the *WRN* gene were described (1). Using the present RT–PCR strategy mutations were readily found in various locations within the gene. The biochemical consequences of these mutations are not known.

All of the *WRN* mutations we have found to date either create a stop codon mutation or cause frameshifts that lead to premature terminations. We have not yet found an amino acid substitution in *WRN* that seems to be responsible for the pathogenesis of WS. It is quite possible that the various truncated WRN proteins may be rapidly degraded, resulting in comparable null mutations and comparable phenotypes. Such altered mRNAs are thought to be degraded via a specific pathway (40). In preliminary experiments, we do observe evidence for reduced levels of *WRN* mRNA expression in WS LCLs with four different mutations.

Identical mutations were found across a variety of ethnic groups, raising the question of potential mutationally susceptible sequences. Although the total number of mutations so far found in the WRN protein is not extensive, candidate sequences for such susceptibility would include nt 3677–3920, nt 1336–1395 and nt 2319–2320.

Three instances of compound heterozygous mutations were found: KUN (Japanese), AUS (Caucasian) and SUG (Caucasian). There have been numerous reports of compound heterozygotic

mutations in 'disease genes' (41,42). However, comparatively few compound heterozygotes have been reported in the genomic instability syndromes. Given the comparatively low prevalence of consanguinity in the USA, clinicians should therefore be alert to the diagnosis of WS in the absence of a history of consanguinity. Our experience suggests that WS is underdiagnosed in the USA.

MATERIALS AND METHODS

Samples

WS patients were from an International Registry of Werner Syndrome (George M. Martin, MD, Junko Oshima, MD, PhD, Amy Jarzebowicz, BS). Diagnostic criteria were previously described (18). This study was approved by the University of Washington Institutional Review Board.

RT–PCR

Five µg of poly(A) RNA, isolated from total RNA, using Oligotex (Qiagen Inc.) was reverse-transcribed with random hexamers in 100 µl reaction volume with GeneAmp RNA PCR kit (Perkin Elmer Cetus). Two µl of the RT product were amplified in a 50 µl PCR reaction buffer containing 5 units *Taq* DNA polymerase, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 µM each of dGTP, dATP, dTTP and dCTP. The cycle program was typically: 94°C for 5 min, then 94°C for 45 s, 55°C for 45 s, 72°C for 3.5 min with 2 s increase per cycle for 35 cycles, followed by 72°C for 10 min. Five µl aliquots of the first amplification products were subjected to a nested second amplification in 100 µl reaction volumes. The primer sequences for RT–PCR are listed in Table 1. The secondary PCR products were separated on 1% agarose/1×TBE (100 mM Tris–HCl pH 8.0, 90 mM boric acid and 1 mM ethylenediaminetetraacetic acid) to estimate the concentrations of DNA before sequencing.

Table 1. Primer sequences for the RT–PCR sequencing template

Region of the amplification	1st amplification primers (5′ to 3′)	2nd amplification primers (5′ to 3′)	Size of PCR product
5′ end	GTGGTGGCGCTCCACAGTCATCC CTTTATGAAGCCAATTTCTACCC	AAGACCTGTTGGACTGGATCTTCTC TACTCCAAAATCTCTAAATTTCGG	838
Translation start site to helicase region	GTGGTGGCGCTCCACAGTCATCC CTTTATGAAGCCAATTTCTACCC	TAGGACTTTCAAAGATGAGTG CGTATACAATCCGGTATTTACC	1936
Helicase region	GTGGTGGCGCTCCACAGTCATCC CTTTATGAAGCCAATTTCTACCC	AGATGTACTTTGGCCATTCCAG GCAATGATCCAATCTGGACC	1218
3′ region	GCATTAATAAAGCTGACATTCGCC CGGAAGGCTGATTTAAGATGCC	CATTACGGTGCTCCTAAGGACATG CGGAAGGCTGATTTAAGATGCC	1946

Table 2. *WRN* mutations in Japanese and Caucasian WS patients

Registry no.	Country	Ethnicity	M/F	Location	Mutation	Predicted protein
LGS90610	USA	Caucasian	F	1336	CGA–TGA Arg Stp	368
OW90650	Japan	Japanese	M	1336	CGA–TGA Arg Stp	368
KO90375	Japan	Japanese	M	1336	CGA–TGA Arg Stp	368
KUN9001	Japan	Japanese	M	1336	CGA–TGA Arg Stp	368
				3541–3614	Deletion	1138
AUS40025	Austria	Caucasian	M	1395	A deletion	391
				3691–3803	Deletion	1157
MIM37100	Brazil	Caucasian	F	1509	ATCT insertion	429
SEP9000	Sardinia	Caucasian	F	2319–2320	105 bp insertion	708
SUG17802	USA	Caucasian	M	2320–3056	Deletion	704
				2896	CGA–TGA Arg Stp	888
IB90550	Japan	Japanese	F	3677	A deletion	1160
BLS60010	France	Caucasian	M	3724	CAG–TAG Gln Stp	1164
TUR90010	Turkey	Caucasian	M	3724	CAG–TAG Gln Stp	1164
SYR10006	Syria	Syrian	M	3919–3922	ACAG deletion	1245

Direct sequencing of PCR products

RT–PCR products were sequenced using a T7 sequence PCR product sequencing kit (UBS, Amersham Life Science, Inc.). Seven µl of PCR product was pretreated with 15 U of exonuclease I and 1.5 U of shrimp alkaline phosphatase at 37°C for 15 min followed by inactivation of the enzymes at 80°C for 15 min, then mixed with 100 ng of sequencing primers. The sequencing reaction followed the manufacturer's instructions.

The sequencing gel contained 6.6% LongRanger polyacrylamide (J. T. Baker Inc.), 6 M urea and 1.2× TBE. The running buffer contained 0.6× TBE. The gel was run at 55 W, dried and exposed overnight to Biomax MR film (Eastman Kodak Co.).

ACKNOWLEDGEMENTS

We thank Dr Goberdhan P. Dimri for a human cDNA library, an important contribution leading to the original positional cloning of *WRN*. We also thank Annette Smith, Charles E. Ogburn, Thao Dang, Susan Fredell, Ellen Nemens and Deanne Sparlin for their technical support. This work was supported by National Institute on Aging grants P1 AG08303 (GMM), R37 AG08303 (GMM), T32 AG00057 (GMM), RO1 AG12019 (GDS) and CNPq and FAPESP, Brazil (MIM).

ABBREVIATIONS

WS, Werner syndrome; *WRN*, Werner syndrome gene; UV, ultraviolet; ERCC, excision repair–cross-complementing; LCL, lymphoblastoid cell line; RT–PCR, reverse transcription–polymerase chain reaction; PCR, polymerase chain reaction.

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