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Down-Regulation of the Defective Transcripts of the Werner's Syndrome Gene in the Cells of Patients

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Werner's syndrome (WS), an adult progeria, is a recessive genetic disorder caused by the mutations in the DNA helicase gene (WRN). In this study, a comparative northern blot analysis was made for poly(A)$^+$ RNAs extracted from fibroblasts and B-lymphoblastoid cells of WS patients, relatives of patients, and normal individuals. The levels of mutant WRN mRNA from patient cells were significantly lower than those of intact mRNA from the cells of normal individuals by an average of 70%. Furthermore, an extremely low level of WRN mRNA(s), presumably a mixture of mutant and intact mRNAs, was observed for the patient's family members who carry one mutated allele. These results strongly suggest that a relatively low level of helicase mRNA is sufficient to prevent the onset of Werner's syndrome.

Werner's syndrome (WS) is an autosomal recessive disorder causing symptoms of premature aging, such as white hair, baldness, cataracts, and osteoporosis (1-3) accompanied by rare cancers (4). The causative gene of WS has been identified in the short arm of chromosome 8, which encodes a protein significantly similar to the *E.coli* RecQ-type DNA helicases (5). At least 19 mutations have been identified thus far in WS patients by the group of Drs.Schellenberg and Martin, as well as by us (5-8). The gene is composed of a total of 35 exons and the mRNA (5189 nucleotide residues) coding the1432 amino acid polypeptide (8, 9). All the mutations were in the coding region, almost throughout the entire helicase molecule, and apparently resulted in premature termination of translation by generating nonsense codons. Fibroblasts and B-lymphoblastoid cell lines transformed by the Epstein-Barr virus (EBV) are two major sources of cells from patients that are available for cellular studies. The WRN mutation is known to cause various abnormal phenotypes in these cells, such as the shortening of the maximal population doubling levels (PDLs) (10), chromosomal aberration called variegated translocation mosaicism (11), and abnormal telomere dynamics (12). Little, however, is known about the molecular mechanisms whereby the WRN helicase gene mutation causes these abnormalities or about the metabolism of the transcripts of the mutated gene.

We and others showed by RT-PCR or northern blot analysis that the mRNA of the WRN helicase gene was transcribed in both fibroblasts and EBV-transformed B-lymphoblastoid cell lines (5,9). In this study, we show that the amounts of mutant WRN mRNA are reduced in these cells having either homozygous or heterozygous mutation.

MATERIALS AND METHODS

The patients were diagnosed as previously reported (3). Because patients with any of the three mutations 1, 4 or 6 comprised about 75% of the total Japanese patients (10), we used the cells with these mutations. Fig. 1 shows the positions of these mutations in the cDNA, and the list of patients and normal individuals from whom the cell lines were obtained. Mutation 1 is the change of one base (C → T) at nucleotide 4144 (in the 33rd exon) in the coding region generating a new stop codon of TGA at the arginine codon of CGA at amino acid residue 1305; mutation 4 is a single base change (G → C) at the splice donor sequence at the intron flanking the 26th exon, resulting in abnormal splicing ; mutation 6 is a C → T substitution at nucleotide 1336 (in the 9th exon) of the cDNA, generating a stop codon of TGA at amino acid residue 369 (5, 7). The fibroblasts were obtained from human biopsy skin samples (2), and B-lymphoblastoid cell lines were obtained by transforming peripheral blood leukocytes with EBV (13). Poly(A)$^+$ RNA was extracted from fibroblasts (2×10^7 cells) and EBV-transformed B-lymphoblastoid cells (4×10^7 cells) using the AGPC (acid guanidinium thiocyanate-phenol-chloroform extraction) method (14) and oligotex^{TM}-dT30 (15). Three μg of poly(A)$^+$ RNA were electrophoresed in 1% agarose gels containing formamide and

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Abbreviations: WS, Werner's syndrome; WRN, Werner's syndrome gene; EBV, Epstein-Barr virus; PDLs, population doubling levels; GTF2E2, general transcription factor 2E β-subunit.

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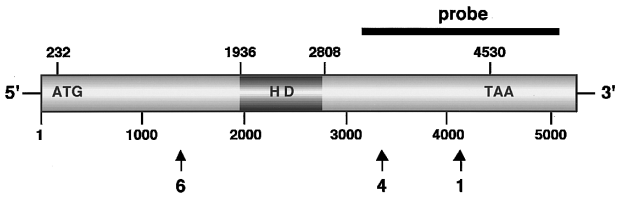

Subject No.	Age/Sex	Mutation	Diagnosis
N0006	29 / M	– / –	Normal
N0007	35 / M	– / –	Normal
N0008	51 / M	– / –	Normal
WS10001	46 / M	1 / 1	Patient
WS0801	42 / F	4 / 4	Patient
WS9801	42 / M	4 / 4	Patient
WS10801	38 / F	4 / 4	Patient
WS10201	43 / F	6 / 6	Patient
WS11001	38 / F	6 / 6	Patient
WS0101	30 / M	1 / 4	Patient
PN0102	27 / F	1 / –	Normal
PN0104	60 / F	4 / –	Normal

FIG. 1. Position of mutations 1, 4, and 6 in the cDNA of WRN helicase and the list of patients and normal individuals from whom the cell lines were obtained. N, WS, and PN indicate normals, Werner's syndrome patients, and phenotypically normal subjects, respectively.

were transferred to Hybond-N membranes (Amersham). Hybridization was performed with a 2×10^6 cpm/ml ^{32}P-radiolabelled probe prepared from the C-terminal region, which includes the 3′-untranslated region (nucleotide residues 3199-5065) of the WRN gene (Fig.1), at 42°C for 16 h. Then the membranes were washed in 0.2 × SSC / 0.1% SDS at 42°C for 30 min and autoradiographed for five days. For quantitative analysis, the membranes were stripped and then were rehybridized with a ^{32}P-radiolabelled β-actin probe as a control to normalize the density. The relative intensities of individul WRN mRNA bands were estimated by a BAS-1500 Bioimaging Analyzer (Fujifilm), assuming the intensity of normal to be 100%.

RESULTS AND DISCUSSION

The levels of WRN gene transcripts were compared in both fibroblasts and B-lymphoblastoid cell lines obtained from a normal individual (N0006) and a WS patient (WS10801). The WS10801 patient carries the homozygous mutation 4. Similar amounts of poly(A)$^+$ RNA extracted from cell lines at a similar PDL cultured under the same conditions were analyzed by northern blot analysis using a ^{32}P-labelled DNA. Fig. 2A shows that the WRN gene transcript level from the WS patient was much less than that from the normal individual, for either fibroblasts or B-lymphoblastoid cells. The expression levels of β-actin mRNA assessed as a control were almost the same for normal and WS cells. The larger transcripts of about 8 kb or greater could be the stable intermediate products of the splicing reaction.

We used B-lymphoblastoid cell lines to investigate this finding more systematically. Since B-lymphoblastoid cells grow faster than fibroblast cells, they allowed us to compare the mRNA levels at almost the same PDLs, as well as to obtain the cells from a greater numbers of patients whose mutation sites were defined. In addition, using amenable B-cells enabled us to extend the study to the normal relatives of heterozygous patients who carry only one allele of the mutated gene. The B-cells with homozygous mutation 4 (WS0801 and WS9801) or mutation 6 (WS10201 and WS11001) had much less mRNA than the normal cells (N0007 and N0008) at PDLs between 48 and 87. On average, the amount of mRNA of patients was about 30% of that of normal individuals, meaning that the cells with a mutation in only one allele should have about 65% (i. e. 50% + 30%/2) of normal cell mRNA. The tailing in both the short and long transcript regions was more obvious in the patient cells than in the normal cells (Fig. 2B, lanes 3 and 5), suggesting an augmented degradation of the mRNA and/or abnormal splicing in patient cells, as shown in other cases in which nonsense codons affected RNA metabolism in vertebrate cells (16,17). To verify that the observed degradation occurred specifically on the WRN mRNA, we examined the levels of GTF2E2 mRNA (whose genomic location is close to that of the WRN gene (18)) (Fig. 2B). The levels of GTF2E2 mRNA were apparently unaffected by the WRN mutation. We also examined three members of a patient family (Fig. 3): a phenotypically normal mother (PN0104) with heterozygous mutation 4 (lane 3), a phenotypically normal sister (PN0102) with heterozygous mutation 1 (lane 2) and a patient with compound heterozygous mutations 1 and 4 (lane 1). The patient cells showed a very low level of the WRN mRNA (13% of the normal level) as expected from the result with fibroblast cells shown in Fig.2A. Surprisingly, the cells from the apparent normal relatives having a heterozygous mutation also showed reduced levels of mRNA (37% and 55% of normal level), lower than the theoretical level of mRNA expected to be present in heterozygous cells, that is, 65% mentioned above in this section. The reason(s) behind this reduction in intact WRN mRNA accompanied by the enhanced reduction of defective mRNA is difficult to explain in molecular terms, due to a limited data and knowledge in the research field dealing with the specific degradation of nonsense mRNA. Notwithstanding this enigma, we believe that our finding provides data new to mRNA dynamics with a mixed population of intact and defective mRNA.

In general, the reduced levels of mRNA may be attributed to a reduction in transcription efficiency or mRNA decay. The results of this study do not necessarily exclude the possibility of a reduction in transcription efficiency, but a mutation affecting the transcription efficiency of the WRN gene is unlikely because the levels of GTF2E2 mRNA were unchanged between

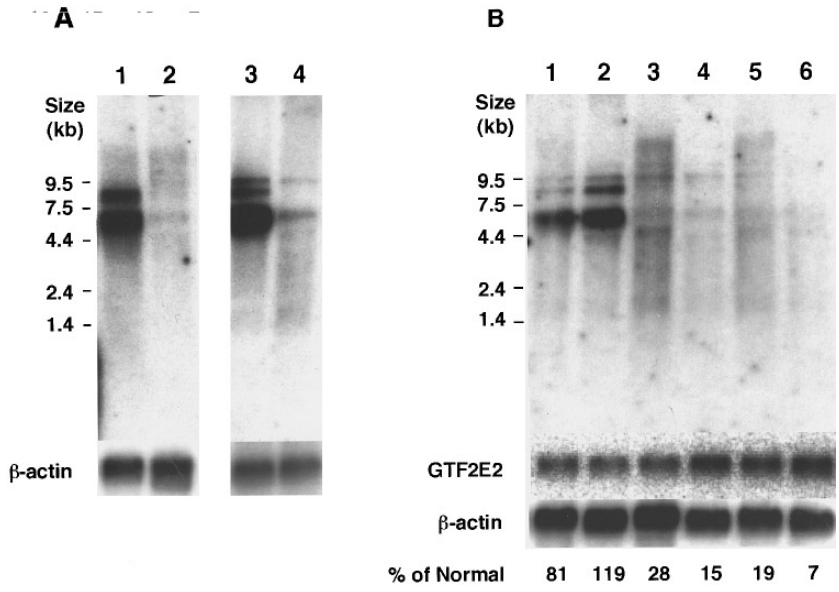

FIG. 2. Northern blot analysis of the cells from normal individuals and WS patients. (**A**) Fibroblasts from a normal individual N0006 (lane 1) and from a patient WS10801 (lane 2) and B-lymphoblastoid cells from a normal individual N0006 (lane 3) and from WS10801 (lane 4). (**B**) B-lymphoblastoid cells from normal individuals N0007 (lane 1) and N0008 (lane 2) and from patients WS0801 (lane 3), WS9801 (lane 4), WS10201 (lane 5), and WS11001 (lane 6). The β-actin and GTF2E2 mRNAs on the same filters were hybridized by specific probes and were used to compare and normalise the data.

normal and patients (Fig. 2B). Rather, the diffusion of mutated mRNA into smaller sizes in the northern blot analysis (Fig. 2B, lanes 3 and 5) is consistent with the second possibility that the degradation of mutated mRNA is enhanced, as with the mutated β-globin and triose phosphate isomerase genes (16, 17). What would then be the biological benefit for the down-regulation of mutated transcripts? The accumulation of defective mRNA may have the following two disadvantages: 1) translation of defective proteins is not economical to the cells, and 2) with heterozygotes, those proteins are harmful due to the competition with normal products.

Recently, we found that most of the mutations in the WRN gene generate the premature termination of translation, resulting in the impaired nuclear transportation of gene products in the cells of patients (19). The molecular events associated with the WRN mutations may thus be a down-regulation of transcripts (shown in this paper), the generation of truncated protein and their impaired nuclear transport.

This study showed the clinically important conclusions that 1) WRN mRNA levels were significantly reduced in patient cells, 2) the WRN mRNA expressed by a normal allele of a heterozygous individual was down-regulated by an unknown mechanism(s), but 3) a relatively low level of helicase mRNA was sufficient to prevent the onset of Werner's syndrome.

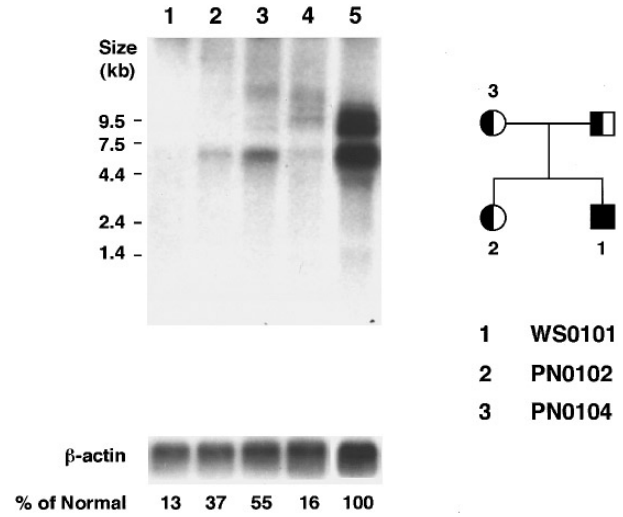

FIG. 3. Northern blot analysis of the cells from the family of a patient WS0101 with compound heterozygous mutations 1 and 4. B-lymphoblastoid cells from patient WS0101 (lane 1), his normal sister PN0102 with a heterozygous mutation 1 (lane 2), and his normal mother PN0104 with a heterozygous mutation 4 (lane 3). The profiles of patient WS10001 (lane 4) and a normal individual N0006 (lane 5) are shown as reference.

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