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IDENTIFICATION OF MUTATIONS IN DNA POLYMERASE ß mRNAs FROM PATIENTS WITH WERNER SYNDROME*

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Summary: Werner syndrome (WS) is a rare autosomal recessive disorder characterized by prematurely aged appearance. Genetic linkage analysis has placed the relevant gene in subchromosomal band 8p12. DNA polymerase ß gene has been mapped to chromosome 8p12-11 and thought to be involved in DNA repair and possibly in recombination. Somatic cells from WS patients exhibit chromosomal instability, a markedly reduced replicative life span and slow growth. The functions of DNA polymerase ß gene and its position prompted us to examine this gene in WS patients. We have found the novel DNA polymerase ß cDNA species in blood samples from WS patients, which contain 107 bp insertions or 87 bp deletions in the catalytic domain of DNA polymerase ß. These mutations change the structure of DNA polymerase ß and thus the capacity of the DNA repair system would be impaired, which may account for the high mutation rate observed in WS.

Werner syndrome (WS; Mckusick catalog number 27770) is a rare, autosomal recessive disease which manifests symptoms of significantly accelerated aging, including graying or loss of hair, sclerosis, hyperkeratosis, cataracts, diabetes mellitus, atherosclerosis and high frequency of neoplasia (1).

Skin fibroblasts from WS patients show a greatly reduced replicative life span and slow growth compared to that of cells from age-matched normal individuals (2). WS lymphoblastoid cell lines also grow poorly (3). The chromosome abnormalities in both fibroblast and lymphocyte cultures of WS patients consist predominantly in stable structural chromosome rearrangements ranging from partial chromosome deletions to multiple translocations (3). Cells obtained from patients with WS were also found to show elevated rates of mutations (especially deletions) of the hypoxanthine phosphoribosyl transferase (HPRT) locus (4) and an increased rate of homologous recombination has been suggested (5). Despite a number of cytogenetic and biochemical studies, the genetic defect in WS has not been identified.

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Positional cloning is therefore used to investigate the disorder and genetic linkage analysis has placed the WS locus between the genetic markers ANK1 and D8S87 on chromosome 8p (6). Thomas,W. et al., presented comprehensive chromosome 8 linkage maps that suggested the order of the mapped loci to be 8pter-D8S135-D8S87-HRG (heregulin)-WT251-WS-ANK1-PLT-8cen (7). We have considered DNA polymerase β gene as a potential candidate locus of WS, as DNA polymerase ß gene resides on chromosome 8p11-12 and is involved in DNA repair (8) and possibly in recombination (9).To test this hypothesis, the whole coding region of DNA polymerase ß mRNA from WS patients and healthy individuals were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR), cloned and sequenced. Here, we have described novel DNA polymerase ß cDNA species from WS patients which contain insertions or deletions in the catalytic domain of the enzyme.

Materials & Methods

Blood samples: Blood samples were obtained with informed consent. WS1 and WS2 patients were female WS cases from two independent families. Samples were collected from the WS1 family members, WS1 and WS2 patients. Neither of these families were consanguineous.

RT-PCR and DNA sequencing: Total RNA was isolated by acid-guanidinium thiocyanate-phenol-chloroform (AGPC) method (10). For RT-PCR, first strand cDNA was synthesized from 1 μg of total RNA following the instruction of cDNA Synthesis System Plus (Amersham). PCR was performed with the reaction mixture 100 μl containing 10 mM Tris-HCl(pH9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 100 pmole primers, 2.5 units of *Taq* DNA polymerase (Promega) and a thermal cycler (Program Temp Control System PC-700, Astech). Each cycle consisted of incubation at 94 °C for 1 min, followed by 2 min annealing at 60 °C and 2 min extension at 72 °C. Before the first cycle, the samples were incubated for 4 min at 94 °C, and after 35 cycles, the extension at 72 °C was prolonged to 5 min. A pair of primers, ß1: ATGAGCAAACGGAAGGCGCC and ß18: TCATTCGCTCCGGTCCTTG were designed to amplify the whole coding sequence of human DNA polymerase ß cDNA (1008 base pairs) (11). The second pair of primers, ß10: GCTACAGTCTGTGGCAGT and ß11: CACCACTGGACTCTGCAC were used to confirm the observed DNA polymerase ß insertions in the catalytic domain.The PCR product was separated on 3 % NuSieve 3:1 agarose gel and stained with ethidium bromide. After phenol and chloroform extraction, the PCR products were blunted using the Blunting kit (Takara), and cloned into *Eco*RV digested Bluescript KS vector (Strategene). Plasmids with DNA polymerase ß insert were isolated from transformed *Escherichia coli* JM109 by alkaline miniprep and sequenced by dideoxy method (12) using a Sequenase 2.0 sequencing kit (USB) and α-^{32}PdCTP (Amersham). RT-PCR products using primers ß10 and ß11 were extracted with chloroform and 5 μl aliquots were separated by 1 % agarose gel, transferred to Hybond N filter (Amersham).

Results

We first looked for gross rearrangement or deletion of the DNA polymerase ß gene by hybridization of sequences representing the entire coding sequence to twenty restriction enzymes digested DNA from WS patients and healthy individuals. No differences in banding pattern between WS patients and healthy controls were observed when all of the restriction enzymes were tested. Therefore, we decided to look for differences by

sequencing the complete open reading frame of the DNA polymerase ß gene from affected and unaffected individuals.

Reverse-transcribed, polyadenylated RNAs from two unrelated WS patients and healthy control blood samples were subjected to PCR amplification using pairs of oligonucleotide primers derived from the reported cDNA sequence (11. 13) of the DNA polymerase ß gene (Fig.1). PCR products were analyzed by electrophoresis through 3% NuSieve 3:1 agarose gel and detected by staining with ethidium bromide. Amplification between primers ß1 and ß18 produced a expected 1 kilobase fragment (A) and a smaller fragment (B) from healthy controls (Fig.2, lanes 5 and 6). In addition to these bands, the PCR reactions from two unrelated WS patients, using primer combinations ß1 and ß18 yielded a slightly larger one (C) than expected (Fig.2, lanes 1 and 4). Trace amounts of this larger band were observed for healthy subjects.

To investigate whether these changes could be related to the development of WS in WS patients, we extended the analysis to other members of WS1 family (WS1 patient's father was died of stomach cancer). Using primers ß1 and ß18, a slightly larger PCR product (C) was observed for WS1 mother and not for WS1 sister (Fig.2, lanes 2 and 3). WS1 sister was 40 years and did not develop any clinically detectable symptoms of WS. WS1

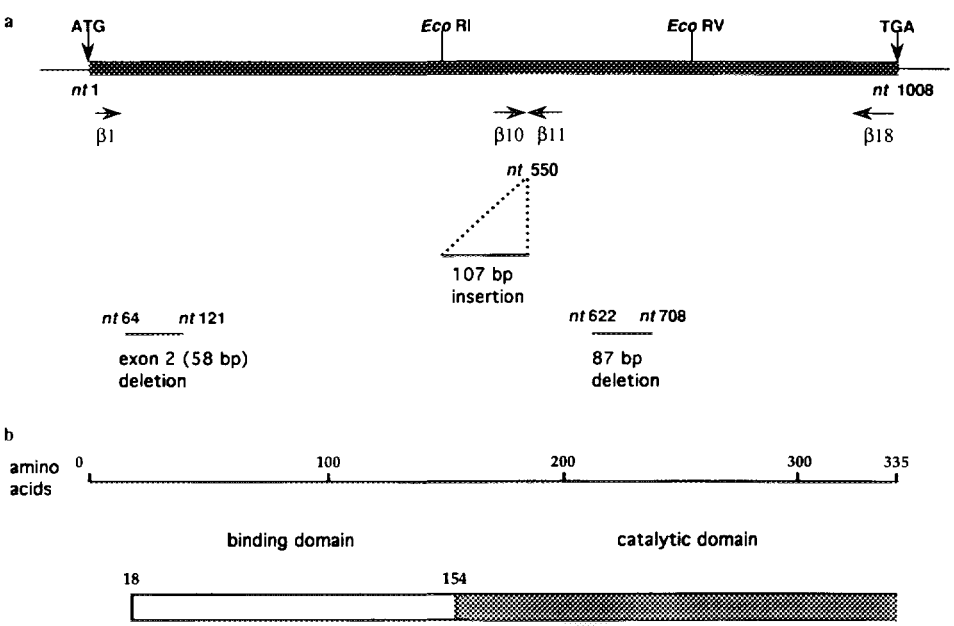

FIG. 1. Schematic diagram of novel DNA polymerase ß cDNA species. (a) The open reading frame (black box) and relevant restriction sites of the published DNA polymerase ß cDNA (11) are shown in the top line. Numbers below the black box refer to nucleotide (nt) positions in the cDNA sequence. Location and orientation (arrowheads) of the primers used in PCR of DNA polymerase ß cDNA and location of insertion or deletion of novel DNA polymerase ß cDNA species are indicated below.
(b) Domain structure of the DNA polymerase ß protein showing template binding (white box) and catalytic (hatched box) domains (18, 19).

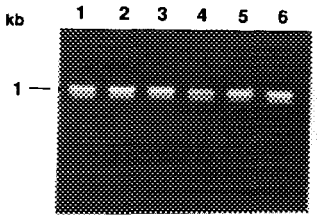

FIG.2. PCR using primers ß1 and ß18 of DNA polymerase ß cDNA. PCR products were separated by 3% NuSieve 3:1 agarose gel electrophoresis. Lanes 1, WS1 patient; 2, WS1 carrier mother; 3, WS1 unaffected sister; 4, WS2 patient;and 5, 6, healthy controls.

mother had a reduced amount of this abnormal fragment in comparison with WS1 patient. To characterize these novel cDNA species, the PCR products from affected and unaffected individuals were cloned and sequenced by dideoxy method.

The differences found in the novel cDNA species compared to the sense strand of a section of DNA polymerase ß cDNA are summarized in Fig.3. The slightly larger fragments (C) obtained with primers ß1 and ß18 from WS1, WS2 patients and WS1 mother contained a 107 bp insertion compared with the published cDNA sequence of

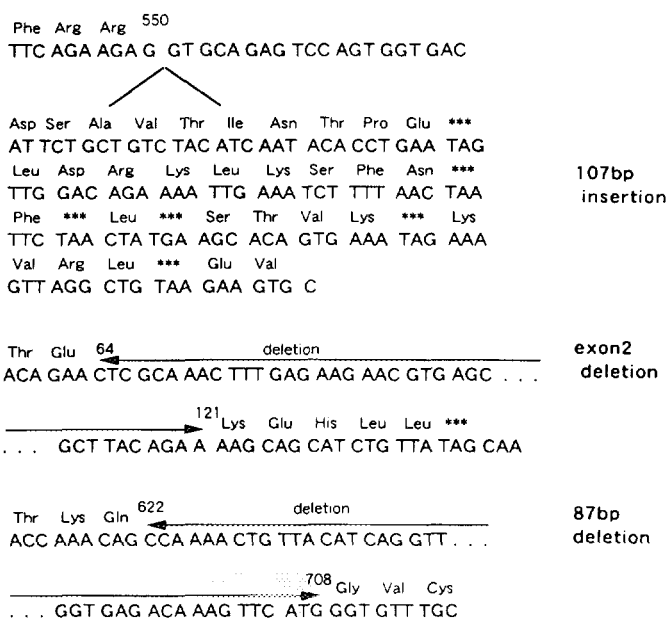

FIG.3. Nucleotide sequences and deduced amino acid sequences of three novel DNA polymerase ß cDNA species. A section of reported DNA polymerase ß cDNA sequences (11) is shown at the top. Stop codons are indicated by three asterisks. Numbers above the nucleotide sequences refer to nucleotide positions, 1 taken as the first nucleotide of the translation initiation codon of DNA polymerase ß gene. 107 bp insertions (top line) were observed in WS1 and WS2 patients, 87 bp deletions(bottom line) in WS2 patient, and exon 2 (58bp) deletion (middle line) in both WS affected and unaffected individuals.

DNA polymerase ß. This insertion altered the reading frame of the mRNA and stopped at a TAG after 10 amino acids and predicted a truncated peptide of 193 amino acids. The sequence of the expected 1kb PCR fragments (A) from affected and unaffected subjects matched that of the reported cDNA.

The smaller fragment (B) could be derived from a deletion of 58 bp corresponding to exon 2 (13). Deletion of this exon caused the reading frame shift which produced a smaller DNA polymerase ß consisting of only 26 amino acids. This smaller PCR fragments were found much more in WS2 patient than in healthy controls (Fig.2, lane 4). Furthermore, another 87 bp deletion was observed with cDNA in WS2 patient. This mutation abolished amino acids 208 to 236 but did not affect the reading frame. Fig.4 shows the results of the PCR reactions using primer pair ß10 and ß11 flanking the inserted area instead of ß1 and ß18. Since the PCR products could not be distinguished from primers complex, we analyzed these products by Southern blot. ^{32}P-labeled inserted DNA sequence hybridized with PCR products from WS1 patient, her carrier mother, and WS2 patient, but not with those from WS1 unaffected sister and healthy controls.

Sequence homology searches using GENBANK indicated that this inserted DNA sequence showed significant nucleic acid homology with yeast RADH gene for putative helicase (58%) (14), 3'-end of mouse calmodulin mRNA (61.7%) (15), human immunodeficiency virus type 1 (60.6%) (16).

Discussion

We have identified the novel DNA polymerase ß cDNA containing a 107 bp insertion in two independent WS cases. This change was also observed in WS1 carrier mother's cDNA as expected from an autosomal recessive inheritance, but not in WS1 unaffected sister and healthy population. The 107 bp insertion could yield a truncated protein with novel 10 carboxy-terminal amino acids deleted for 152 residues. WS2 patient had also another abnormal fragment which deleted amino acids 208 to 236 (29 residues). It is important to note that this mutation is consistently found in colorectal carcinomas (17).

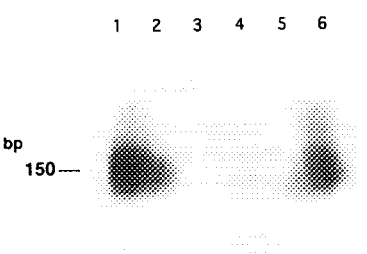

FIG.4. Southern blot analysis of RT-PCR products from blood samples using primers ß10 and ß11. The inserted DNA sequence was used as the probe. Lanes 1, WS1 patient; 2, WS1 mother; 3, WS1 sister; 6, WS2 patient; and 4, 5, healthy controls.

Proteolytic and chemical cleavage studies indicate that DNA polymerase ß is organized in two functionally distinct domains: the amino-terminal segment (about 75 amino acids) contributes single-stranded (ss) nucleic acid binding, and the carboxy-terminal segment (about 250 amino acids) contributes catalytic activity and double-stranded (ds) nucleic acid binding (18). The segment from residues 18-154 of DNA polymerase ß is able to bind both ss and ds nucleic acid lattices (19). Aspartic acid (Asp) residues which are involved in the interaction with primer are located at positions 190 and 192 in the catalytic center of the enzyme (20). As shown in this paper, the insertions and deletions occurred at a region adjacent to these two Asp residues of DNA polymerase β. The mutations would generate an impaired DNA polymerase β which is incapable of repairing the lesion and might account for the high mutation rate observed in WS (4). Deletion of exon 2 is unlikely to be associated with the development of WS, since this fragment was observed in both affected and unaffected individuals. This deletion would produce a truncated protein with only 26 amino acids, in which both the DNA binding domain and the catalytic domain of DNA polymerase ß should be missing.

In combination with the current data on genetic map of the WS locus, our results suggest that the mutated DNA polymerase ß may have a key role in WS. However, in the absence of studies that demonstrate impairment of DNA polymerase ß function in affected subjects, this hypothesis needs to be confirmed by the detection of mutations in the DNA polymerase ß gene in other WS cases and more detailed genetic linkage map of chromosome 8p.

Are the insertion and deletion events due to abnormal splicing or any rearrangement of the genome or the artifact of PCR? As one suggestive example for abnormal splicing, Rosenthal, A. et al reported the alternation of neural cell adhesion molecule L1 mRNA in X-linked hydrocephalus (21). They have identified a single intronic A to C base change 19 bp upstream of a splice acceptor site in the L1 gene that caused novel L1 mRNA species containing deletions and insertions in addition to normal L1 mRNA. Alternatively, these mutations may simply be caused by chromosomal rearrangements, since somatic cells from WS patients reveal a propensity to develop chromosomal aberrations, including translocations, inversions, and deletions (3). To elucidate these questions and the interrelationships between WS and DNA polymerase ß, we are now studying the appropriate DNA polymerase ß regions of genomic DNAs from WS family members and healthy individuals.

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