A high prevalence of myeloid malignancies in progeria with Werner syndrome is associated with p53 insufficiency

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Werner syndrome (WS) is a progeroid syndrome caused by mutations in the *WRN* gene, which encodes the RecQ type DNA helicase for the unwinding of unusual DNA structures and is implicated in DNA replication, DNA repair, and telomere maintenance. patients with WS are prone to develop malignant neoplasms, including hematological malignancies. However, the pathogenesis of WS-associated hematological malignancies remains uncharacterized. Here we investigated the somatic gene mutations in WS-associated myelodysplastic syndrome/acute myeloid leukemia (MDS/AML). Whole-exome sequencing (WES) of 4 patients with WS with MDS/AML revealed that all patients had somatic mutations in *TP53* but no other recurrent mutations in MDS/AML. *TP53* mutations were identified at low allele frequencies at more than one year before the MDS/AML stage. All 4 patients had complex chromosomal abnormalities including those that involved *TP53*. Targeted sequencing of nine patients with WS without apparent blood abnormalities did not detect recurrent mutations in MDS/AML except for a *PPM1D* mutation. These results suggest that patients with WS are apt to acquire *TP53* mutations and/or chromosomal abnormalities involving *TP53*, rather than other MDS/AML-related mutations. *TP53* mutations are frequently associated with prior exposure to chemotherapy; however, all four patients with WS with *TP53* mutations/deletions had not received any prior chemotherapy, suggesting a pathogenic link between *WRN* mutations and p53 insufficiency. These results indicate that WS hematopoietic stem cells with *WRN* insufficiency acquire competitive fitness by inactivating p53, which may cause complex chromosomal abnormalities and the subsequent development of myeloid malignancies. These findings promote our understanding of the pathogenesis of myeloid malignancies associated

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H. Kato, AI, MS, and KY designed this study. H. Kato and YM performed experiments and actively wrote the article. DN, EN, JT, TK, YS, SM, and SO analyzed next-generation sequencing data; MY, H. Kaneko, and M. Takemoto collected samples from patients. TM, YT, SI, YH, HN, JI, M. Takeuchi, M. Koshizaka, NM, M. Kuzuya, and ES analyzed clinical data. AI, MS, and KY conceived of and directed the project, secured funding, and actively wrote the article.

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HIGHLIGHTS

- Patients with Werner syndrome are likely to acquire *TP53* mutations/?deletions.
- Patients with Werner syndrome and MDS/AML have complex chromosomal abnormalities.
- Werner syndrome hematopoietic stem cells acquire competitive fitness by inactivating p53.

Werner syndrome (WS) is a progeroid syndrome characterized by the early onset of aging-related symptoms [1,2]. It is caused by mutations in the *WRN* gene on 8p12, which encodes the RecQ type DNA helicase. The WRN helicase belongs to a DEAH box-containing RecQ family of helicases and has a high degree of helicase activity for the unwinding of unusual DNA structures, such as G4 quadruplex sequences and four-way junctions that resemble intermediates of DNA repair and telomere maintenance. It has been hypothesized that WRN plays a role in the resolution of potentially damaging, complex DNA structures that were accidentally formed during various DNA transactions, including replication, recombination, repair, and transcription [2]. Patients with WS usually develop normally until they reach the second decade of life. They do not undergo the pubertal growth spurt during the teen years and begin to suffer from skin atrophy with loss of subcutaneous fat and loss and/or graying of hair in their twenties, which results in an overall aged appearance. Bilateral ocular cataracts and type 2 diabetes mellitus are generally recognized while patients are in their twenties and thirties, followed by a series of common age-related disorders including osteoporosis, gonadal atrophy, atherosclerosis, and various malignancies. The most common causes of death are cancers and myocardial infarctions, at a median age of 54 years. We previously examined 163 patients with WS and found that patients with WS are prone to developing malignant neoplasms, including hematopoietic malignancies, such as myelodysplastic syndrome (MDS) (2.4 %) and multiple myeloma (1.2 %) [3]. An independent study reported that MDS and acute myeloid leukemia (AML) were more frequent in their patients with WS than in our study: 11/131 (8.4%) and 4/131 (3.1%), respectively [4]. However, the pathogenesis of WS-associated hematological malignancies remained uncharacterized. In the present study, we performed for the first time the profiling of somatic gene mutations in WS-associated MDS and AML.

METHODS

Ethics

Biological samples were collected after informed consent was obtained, in accordance with the Declaration of Helsinki. The present study was approved by the Ethics Committee of Chiba University Graduate School of Medicine (Approval No. 1029[973]).

Whole-exome Sequencing

Total DNA was extracted from bone marrow or peripheral blood mononuclear cells using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) and from buccal cells or nails using Gentra Puregene Kits (Qiagen). Whole-exome capture was accomplished by the liquid phase hybridization of sonicated genomic DNA with a mean length of 150−200 bp using the bait cRNA library (SureSelect Human ALL Exon V5/6; Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's protocol. Massive parallel sequencing of the captured targets was performed using a HiSeq 2000/2500 sequencing system (Illumina, San Diego, CA, USA) with the pair end 100- to 124-bp read option, according to the manufacturer's instructions. Candidate somatic mutations were detected using an in-house pipeline EBCall (Empirical Bayesian Mutation Calling; available at: https://github.com/friend1ws/EBCall) [5], in which candidate variations fulfilling the following criteria were included in subsequent analyses: (1) variant allele frequencies (VAFs) $\geq$0.02; (2) number of variant reads $\geq$4; (3) VAFs in blood cells greater than those in control cells; (4) variants located on the nonrepeated region; (5) p value by Fisher's exact test $\leq$0.1; (6) p value by EBCall $\leq$0.001; (7) candidates that were not registered in public or private databases, including dbSNP138, ESP6500, the 1000 Genomes Project as of October 2014, the Human Genome Variation Database, and our in-house database. The following candidates were excluded: (1) ambiguous (unknown) variants; (2) variants that were read-only from one direction. Candidates registered in COSMIC version 70 databases and whose frequency was <0.01 in ESP6500 or the 1000 Genomes Project were rescued. Mapping errors were removed by visual inspection on the Integrative Genomics Viewer (IGV) browser (http://software.broadinstitute.org/software/igv/). Copy number alterations (CNAs) were evaluated on the basis of sequencing data using our in-house pipeline CNACS. The codes for CNACS are available at https://github.com/papaemmelab/toil_cnacs.

Targeted Sequencing

A target enrichment library of the selected genes was prepared using a custom-designed SureSelect XT2 Kit (Agilent Technologies). Briefly, genomic DNA was sheared by mechanical fragmentation (S220, Covaris, Woburn, MA, USA). Fragments were end-repaired, A-tailed, and ligated to the specific adapters. The libraries were amplified with primers specific for the adapters and were then hybridized to the designed biotinylated probes at 65°C for 24 hours. Up to 16 DNA libraries were pooled in the same reaction. The resulting libraries were recovered using streptavidin magnetic beads, and postcapture polymerase chain reaction amplification was performed. Sequencing of the libraries was performed on a HiSeq 2000/2500 sequencing system (Illumina, San Diego, CA, USA). Candidate somatic mutations were also detected using our in-house pipeline EBCall with filtering criteria similar to those used in WES.

Rare Mutation Detection Assay by ddPCR

Droplet digital polymerase chain reaction (ddPCR) was performed on the QX100 Droplet Digital PCR system according to the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA). Pairs of primer and probe for mutated *TP53* and *PPM1D* genes were purchased from Bio-Rad Laboratories (Hercules, CA). DNA samples

Table 1 Patient characteristics

Patient ID	Type of *WRN* mutation (NM_000553)	Sex	Age	WBC (per mL)	Hb (g/dL)	Platelets ($\times 10^4$/mL)	Karyotype
WS_001	c. [1105C>T]; [1105C>T]	Male	56	7,300	13.1	27.8	
WS_002	c. [1105C>T]; [1105C>T]	Female	60	7,500	13.7	24.9	
WS_002 (AML)	c.[1105C>T]; [1105C>T]	Female	66	14,700	10.2	7.1	47, XX, -5, del(5)(q?), −6, −11, −12, del(12)(p?), −13,−13, −14, add(15)(p11.2), −17, −21, +rlx2, +8mar in 5/20 cells; other cells exhibited 44 to 49 chromosomes with incompatible karyotypes
WS_003	c.[1105C>T];[3139-1G>C]	Female	56	6,900	12.2	30.7	
WS_004	c.[2959C>T];[3139-1G>C]	Male	40	7,300	12.5	20.7	
WS_005	c.[3139-1G>C];[3139-1G>C]	Female	60	4,200	10.3	24.9	
WS_006	c.[2959C>T];[2959C>T]	Female	39	10,300	12.6	25.1	
WS_007	c.[3139-1G>C];[3446delA]	Male	51	6,500	15.1	28.2	
WS_008	c.[3139-1G>C];[3139-1G>C]	Female	42	4,000	11.6	25.4	
WS_009 (MDS)	c.[3139-1G>C];[3139-1G>C]	Male	43	3,900	8.4	12.4	44-48, XY, −5, −7, dic(11;12)(p15;p13), −19, +mar1, +mar2, +mar3
WS_010	c.[1105C>T];[3139-1G>C]	Male	53	9,900	9	44.9	
WS_011 (MDS/AML)	c.[1105C>T];[1105C>T]	Female	48	1,500	6.4	4.2	46, XX, del(11)(q?) in 1/20 cells; 44, idem, −1, add(2)(q21), del (5)(q?), der(7)t(7;21)(p11.2;q11.2), −21, der(22)t(1;22)(q12; p11.2)ins(22;?)(p11.2;?) in 2/20 cells; other cells exhibited 43 to 46 chromosomes with incompatible karyotypes
WS_014 (MDS/AML)	c.[3139-1G>C];[3913C>T]	Female	58	1,000	7.4	1.1	44, XX, der(5;17)(p10;q10), −7, del(12)(q?), −13, −15, add(16) (q11.2), −22, +3mar[14]/46, XX[6]

AML=acute myeloid leukemia; *Hb*=hemoglobin; *MDS*=myelodysplastic syndrome; *WBC*=white blood cell count.

were fragmented using restriction enzymes before droplet generation, and template DNA was used at 100 ng per reaction. Patient samples were assayed in triplicate, positive control with single, negative control with duplicate, and nontemplate control with duplicate. Data analysis was performed using the QuantaSoft software (Bio-Rad Laboratories).

Data Accession Numbers

Sequencing data obtained in the present study were deposited in the NBDC database (Accession No. JGAS000271/JGAD000377).

RESULTS AND DISCUSSION

To clarify the pathogenesis of WS-associated MDS/AML, we investigated somatic gene mutations in 4 patients with WS with MDS/AML by whole-exome sequencing (WES) and 9 patients with WS and no obvious hematological disorders by target-capture sequencing. DNA was obtained from bone marrow (BM) or peripheral blood (PB) mononuclear cells. DNA from buccal mucosa or nail was used as normal controls [6]. All patients had typical WRN gene mutations (Table 1).

We first analyzed BM or PB mononuclear cells from 4 patients with WS and MDS/AML (3 MDS/AML and 1 AML) (age range: 43−66 years; average: 53.8 years) (Table 1). WES of these WS patient samples revealed that all patients had somatic mutations in *TP53* (Figure 1; Supplementary Tables E1 and E2, online only, available at www.exphem.com). All patients except WS_002 manifested the loss of heterogeneity (LOH) in the same region (the former was deleted, and the latter was copy number neutral [CNN]-LOH) (Figure 2A). WS _002 had two different *TP53* mutations, suggesting biallelic inactivation of the *TP53* gene (Supplementary Tables E1 and E2). Although other gene mutations were also discovered, no recurrent mutations in MDS and AML were identified, and no other mutations were recurrently identified among these 4 patients with WS (Figure 1, Supplementary Table E1). Copy number analysis using the WES data revealed complex chromosomal abnormalities in all 4 cases (Figure 2A). Karyotype analysis also revealed that all these patients with WS had complex karyotypes (Table 1). We next performed ddPCR analysis to detect *TP53* mutations at high sensitivity in samples from 3 patients collected at pre-MDS/leukemia stages (Supplementary Table E3, online only, available at www.exphem.org). Of note, *TP53* mutations were detectable, albeit at low allele frequencies, at more than 1 year before the MDS/leukemia stage, with the longest case more than 6 years.

We then analyzed PB mononuclear cells from 9 patients with WS and no apparent blood abnormalities, including a sample of WS_002 at preleukemia stage (age range: 39−60 years; average: 50.8 years) (Table 1) by targeted sequencing, which covered 288 known or putative driver genes mutated in myeloid malignancies (Supplementary Table E4, online only, available at www.exphem.org). The average depth of coverage was 363 (195−688). Somatic gene mutations, including representative clonal hematopoiesis of indeterminate potential (CHIP) mutations in *DNMT3A, TET2*, and *ASXL1* [7−9], were not detected. Only a *PPM1D* mutation (S468fs) generally associated with prior cytotoxic chemotherapy [9,10] was identified in a patient (WS_002) at a low VAF of 2.6% (Figure 1, Supplementary Table E5, online only, available at www.exphem.org). WS_002 was a 66-year-old woman who had been diagnosed with WS at 57 years

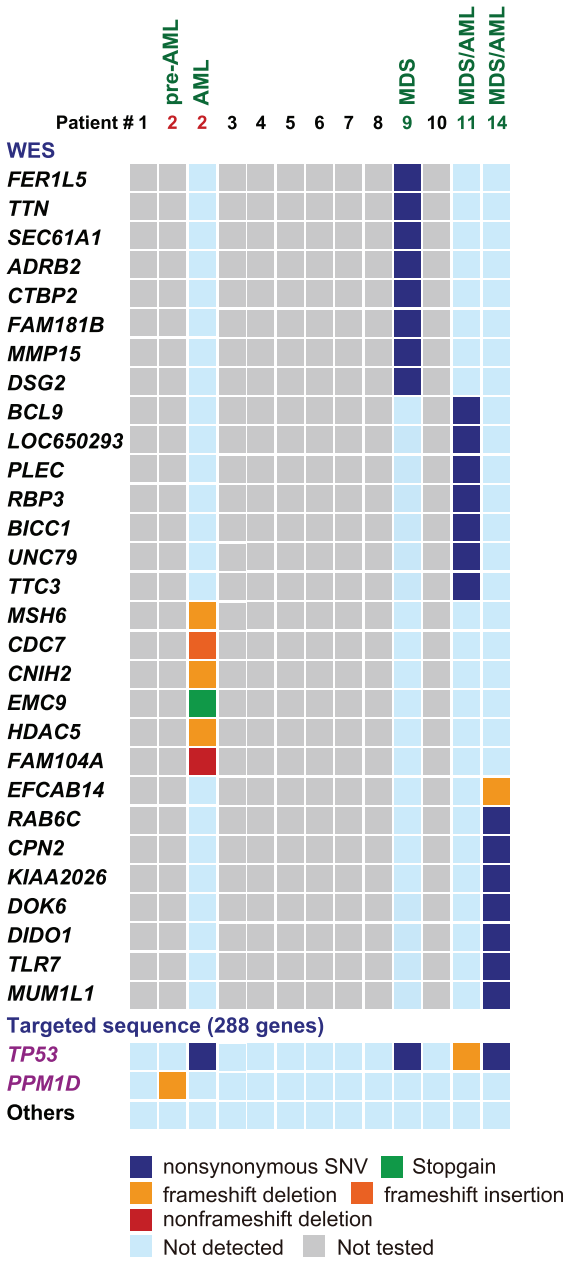

Figure 1 Landscape of somatic mutations in Werner syndrome. The diagram summarizes 31 somatic mutations detected in 33% of cases (4/12) by whole-exome sequencing (patients 2, 9, 11, and 14) and targeted analysis sequencing. *AML*=acute myeloid leukemia; *MDS*=myelodysplastic syndrome; *SNV*=single-nucleotide variant.

of age. At 60 years of age, she was diagnosed with breast cancer and underwent a mastectomy and subsequent aromatase-inhibiting therapy. At 64 years of age, she was diagnosed with lung cancer but did not undergo further examination because of her low performance status. At 66 years of age, 6 years after detection of the *PPM1D* mutation, she developed AML and died. Droplet digital PCR analysis revealed that the allele frequency of *PPM1D* mutation was maintained at a similar level for a long term but profoundly

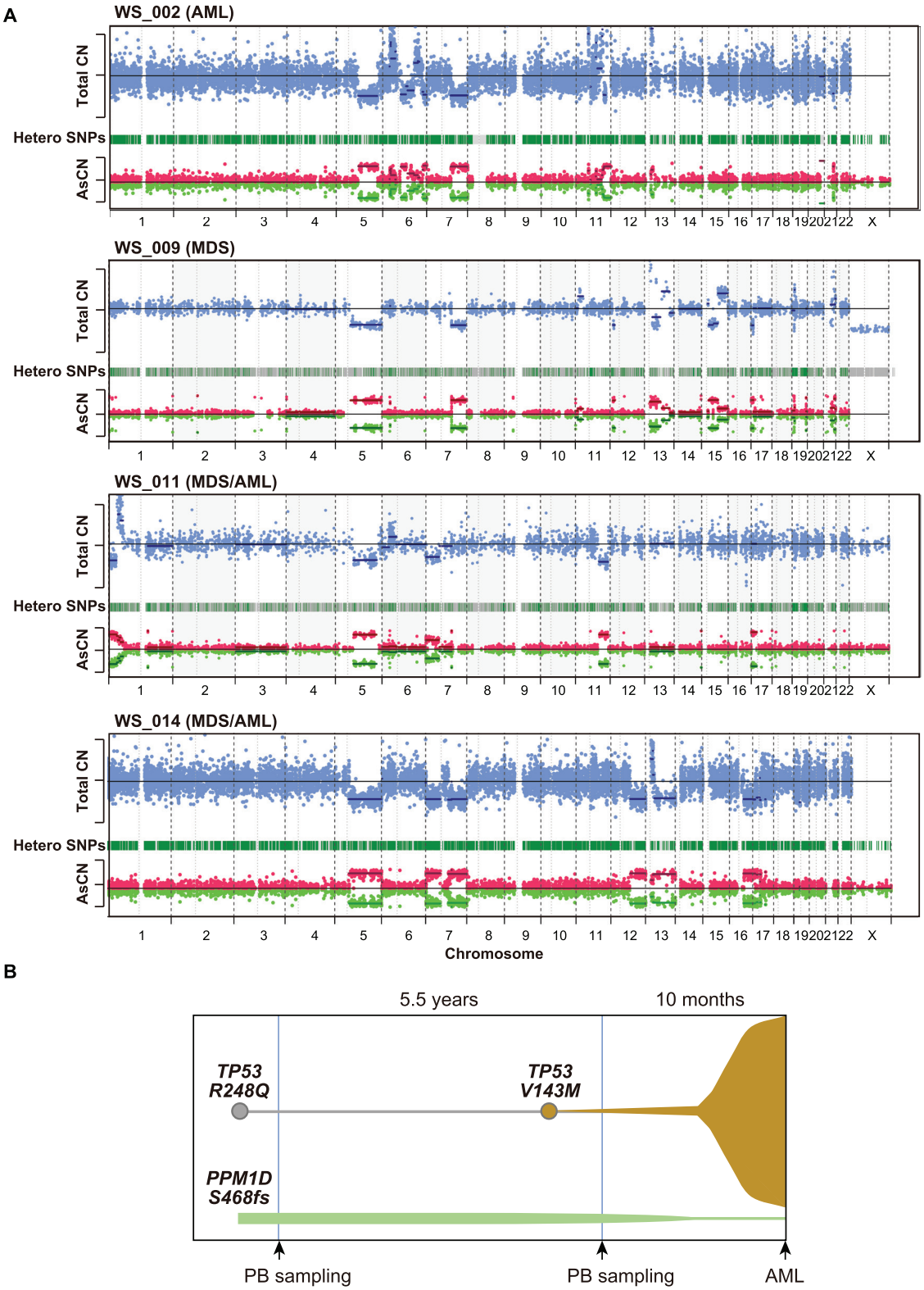

Figure 2 Copy number profiles of PB mononuclear cells in Werner syndrome. **(A)** Copy number analysis of four MDS/AML patients (WS_002, 009, 011, and 014). Allele-specific copy numbers (AsCN) and total copy numbers (CN) are shown. **(B)** Schematic of CH in WS_002. Distinct clones coexisted over time, with only the clone with *TP53* mutations evolving into AML. *AML*=acute myeloid leukemia; *MDS*=myelodysplastic syndrome; *PB*=peripheral blood; *SNP*=single-nucleotide polymorphism.

decreased at AML (Supplementary Table E3), suggesting that AML developed from a clone with *TP53* mutations different from that with a *PPM1D* mutation (Figure 2B).

These results suggest that patients with WS are apt to acquire *TP53* mutations and/or chromosomal abnormalities involving *TP53*, rather than other CHIP-associated mutations, in the development of MDS and AML. In addition to our findings, there are several WS MDS cases with chromosome 17 abnormalities that resulted in the loss of the *TP53* locus at 17p13.1 [11]. *TP53* ranks in the top five among genes that were mutated in CHIP [7–9]. *TP53* mutations are frequently associated with prior exposure to chemotherapy [12]. However, all 4 patients with WS with *TP53* mutations/deletions had not received any prior chemotherapy, suggesting a pathogenic link between *WRN* mutations and p53 insufficiency. Of note, the *PPM1D* mutation in WS_002 at the pre-AML stage encodes the WIP1 phosphatase that is induced by p53-induced cell cycle arrest or apoptosis. WIP1 directly or indirectly dephosphorylates p53, thereby downregulating p53 levels and driving cells back to their normal state [13]. The *PPM1D* mutation in WS_002 was a frameshift mutation (S468fs) that resulted in the overexpression of a truncating WIP1 mutant, leading to the suppression of p53 activity [14]. *PPM1D* mutations, like *TP53* mutations, are associated with prior exposure to specific DNA-damaging agents and have been detected in ~20% of patients with therapy-related MDS or AML [10]. Although the clone with *PPM1D* mutations was eventually outcompeted by the other clone with *TP53* mutations in WS_002, acquisition of *PPM1D* mutation by WS_002 further suggests a pathogenic link between *WRN* mutations and p53 insufficiency.

Although the number of cases analyzed herein was small, the results obtained indicated that WS HSCs are strongly predisposed to p53 insufficiency, which may cause complex chromosomal abnormalities and the subsequent development of myeloid malignancies. WRN is a RecQ-type DNA helicase involved in DNA replication, DNA repair, and telomere maintenance [1,2]. Fibroblasts from patients with WS exhibit premature cellular senescence in vitro [15,16], which is largely dependent on telomere shortening and can be overcome by telomerase overexpression [17,18]. Short telomeres activate p53, leading to cell cycle inhibition, senescence, and/or apoptosis [19]. Deletion of p53 significantly attenuates the adverse cellular and organismal effects of telomere dysfunction, but also increases genomic instability and cancer formation in vivo [19]. In the hematopoietic compartment, activation of p53 causes BM failure as a result of depletion of hematopoietic stem and progenitor cells (HSPCs) [20,21]. The deletion of p21, one of the direct targets of p53, improved the repopulation capacity and self-renewal of HSCs from mice with dysfunctional telomeres, in which the p21 deletion did not accelerate chromosomal instability or tumor formation [20]. *WRN* insufficiency is thus assumed to induce p53 activation, leading to cell cycle perturbation, apoptosis, or senescence. There might be pressure to enhance the competitive fitness of *WRN*-deficient HSPCs by inactivating p53. Mouse embryonic fibroblasts from a mouse model of WS with a double knockout of *mTerc* and *Wrn* rapidly became senescent. Of interest, they spontaneously escaped from senescence and became immortalized. All tumorigenic immortalized cells acquired a single p53N236S point mutation [22], further supporting the link between *WRN* insufficiency and p53 inactivation.

Patients with WS exhibit a unique feature of clonal hematopoiesis that is different from that of normal individuals and rather resembles therapy-related clonal hematopoiesis. In this regard, patients with WS have a feature in common with Shwachman–Diamond syndrome (SDS) patients. SDS is a rare congenital BM failure syndrome with high rates of MDS and AML. All the MDS patients with biallelic *SBDS* mutations had somatic *TP53* mutations, which were significantly more frequent than those with one *SBDS* mutation or no *SBDS* mutation [23]. Correspondingly, clonal hematopoiesis resulting from mutations in *TP53* occurred in 48% of patients with SDS [24]. These data suggest that somatic acquisition of *TP53* mutations mediates the progression to MDS in SDS patients and there might be selective pressure to expand *TP53*-mutated HSPCs in SDS patients. These findings support a biologic synergy between *WRN* mutations and *TP53* mutations in the clonal transformation of HSPCs. Mechanistically, biallelic germline *SBDS* mutations in SDS result in impaired ribosome maturation, translational inefficiency, and p53 activation leading to cellular senescence. *TP53* inactivation enhances the competitive fitness of *SBDS*-deficient cells by inactivating senescence pathways without correcting the underlying SDS ribosome and translational defects [25].

The life span of patients with WS is being extended by advances in therapeutic modalities that prevent the progression of cardiovascular diseases, such as statins and new diabetes agents. Instead, MDS is becoming one of the critical diseases that determine the life span of patients with WS. In our recent cohort, about 30% of deaths caused by WS during the past decade had lethal MDS/AML. Therefore, the pathogenic relationship between *WRN* insufficiency and *TP53* mutations needs to be clarified to develop therapeutic strategies to realize further improvement of the quality of life of patients with WS.

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