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Genetic Instability and Hematologic Disease Risk in Werner Syndrome Patients and Heterozygotes[1]

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ABSTRACT

Werner syndrome (WRN) is an uncommon autosomal recessive disease in which progeroid features are associated with genetic instability and an elevated risk of neoplasia. We have used the glycophorin A (GPA) somatic cell mutation assay to analyze genetic instability *in vivo* in WRN patients and heterozygotes. GPA variant frequencies were determined for 11 WRN patients and for 10 heterozygous family members who collectively carry 10 different *WRN* mutations. Genetic instability as measured by *GPA* Ø/N allele loss variant frequency was significantly increased, and this increase was strongly age-dependent in WRN patients. *GPA* Ø/N allele loss variants were also significantly elevated in heterozygous family members, thus providing the first evidence for *in vivo* genetic instability in heterozygous carriers in an autosomal recessive genetic instability syndrome. Our results and comparable data on other human genetic instability syndromes allow an estimate of the level of genetic instability that increases the risk of human bone marrow dysfunction or neoplasia.

INTRODUCTION

WRN[3] (MIM no. 277700) is an uncommon autosomal recessive disease that results from mutational inactivation of a human RecQ helicase protein encoded by the chromosome 8p *WRN* locus (1). Considerable interest has focused on the WRN phenotype, which resembles premature aging and includes genetic instability and an elevated risk of neoplasia (2, 3). To further examine the role of genetic instability in WRN pathogenesis (4, 5), we have used the GPA somatic cell mutation assay to quantify and characterize erythroid lineage genetic instability and to relate genetic instability to disease risk in WRN patients and heterozygotes.

The GPA assay quantifies somatic mutation by measuring the frequency of GPA variant peripheral blood red cells. *GPA* is a chromosome 4 locus that encodes the M/N blood group antigens. M and N allele GPA variant red cells that arise by mutation are detected and quantified by flow cytometric analysis of M/N-heterozygous peripheral blood erythrocytes that have been immunolabeled with M and N allele-specific monoclonal antibodies (6). GPA Ø/N and M/Ø variant red cells can arise by *GPA* gene mutation, by chromosome 4 loss, or by the epigenetic silencing of *GPA* alleles. GPA N/N and M/M variant red cells that have lost expression of one allele and express the retained allele at a homozygous level may arise by mitotic recombination, by gene conversion, or by chromosome missegregation or loss and then reduplication (Fig. 1 and Ref. 7). GPA variant frequencies (V_f are reported as the number of variant red cells per 10^6 erythrocytes. N allele (Ø/N and N/N) V_f are most often reported because they can be reliably determined at frequencies as low as 1 in 10^6 (6).

The GPA assay has been well-validated as a robust and quantitative assay for somatic mutation (6, 7). Background or spontaneous GPA V_f parallel mutation frequencies determined using the X-linked *HPRT* and autosomal *TCR* and *HLA* loci (reviewed in Ref. 8). GPA V_f increases with age, and an increased GPA V_f has been observed in cigarette smokers (8–10). Patients exposed to ionizing radiation or receiving chemotherapy show GPA V_f elevations that parallel *HPRT* or *HLA* mutation frequency increases. These induced GPA V_f elevations are dose-dependent, and in many instances decay with kinetics that parallel erythrocyte turnover (for example, see Refs. 11 and 12).

Patients with heritable genetic instability syndromes have persistently elevated GPA V_f. Syndromes that have been analyzed include ATM (MIM no. 20890; Refs. 13 and 14), BLM (MIM no. 210900; Refs. 9 and 15), and FAN (MIM nos. 227645, 227646, 227650, 227660, 600901, 603467, and 603468; Refs. 16 and 17). In each of these syndromes, the pattern of GPA V_f elevation is consistent with our understanding of the mechanistic basis for genetic instability. For example, the high level of N/N GPA variants in BLM patients is consistent with the idea that BLM modulates mitotic recombination (18). The availability of GPA V_f data on large numbers of control donors facilitates these types of patient, disease-specific, and population-based analyses (10, 19).

Our results document genetic instability *in vivo* in WRN patients and, surprisingly, in *WRN* heterozygotes. These analyses used red cells from patients and heterozygotes carrying 10 different *WRN* mutations, including two mutations independently reported to lead to an elevated GPA V_f (20). We have also been able to use our data on WRN and comparable results from the study of other genetic instability syndromes to estimate the level of genetic instability that confers a high risk of human bone marrow dysfunction or neoplasia.

MATERIALS AND METHODS

Werner Pedigree and Control Blood Samples. A total of 90 blood samples collected by the International Registry of Werner Syndrome at the University of Washington (21) were analyzed. Samples were serotyped for MN blood group antigens using commercial anti-M and anti-N typing sera (Ortho Diagnostics, Raritan, NJ) according to the manufacture's protocol. Of the 90 samples, 28 (31%) were M/N heterozygous and informative. Genetically characterized blood samples from 22 M/N heterozygous donors who were WRN patients or family members were used for GPA V_f determinations. *WRN* mutation analyses were performed using nucleic acids isolated from lymphoblastoid cell lines established from each sample (1). Control donors with the same age range as WRN patients (n = 283; ages 21–58) or heterozygotes (n = 362; ages 14–72) were chosen from the Lawrence Livermore National Laboratory (Livermore, CA) employee cohort (19). There was no significant difference in the median or mean ages or in the age distribution of control donors and WRN patients or heterozygotes (Tables 1 and 2 and additional results not shown).

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[3] The abbreviations used are: WRN, Werner syndrome; GPA, glycophorin A; MIM, Mendelian Inheritance in Man database; V_f, variant frequency; ATM, ataxia-telangiectasia; BLM, Bloom syndrome; FAN, Fanconi anemia; OR, odds ratio.

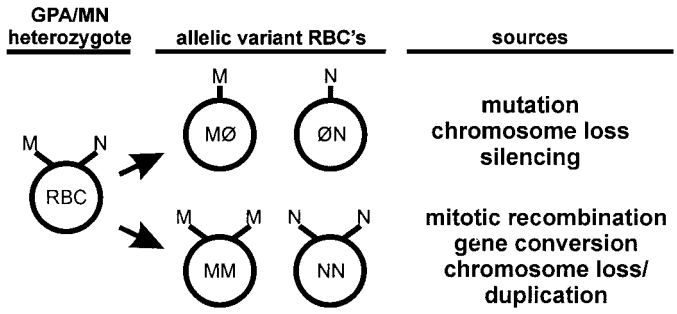

Fig. 1. The GPA V_f assay. The GPA assay uses a combination of immunostaining and flow cytometric analysis to detect and quantify M/N variant erythrocytes that arise by somatic mutation in the erythroid lineage of M/N heterozygous donors. The M/N antigens are encoded by the autosomal *GPA* locus on chromosome 4. *GPA* gene mutations, chromosome loss, or the epigenetic silencing of one *GPA* allele can give rise to M/Ø and Ø/N allele loss variants (*top*, *center* and *right panels*). GPA M/M and N/N variants (*bottom*, *center* and *right panels*) can arise by mitotic recombination, gene conversion, or chromosome 4 missegregation or loss and then reduplication of the remaining chromosome 4. Ø/N and N/N V_f are most often reported because they can be determined at low background frequencies ($\geq$1 in 10^6; Ref. 6).

GPA V_f Determinations. RBC immunolabeling and flow cytometic analysis to determine GPA V_f were performed as previously described (6). In brief, erythrocytes were swollen in hypotonic buffer containing SDS to generate spheroplasts that were fixed with formaldehyde. Fixed cells were immunolabeled with a phycoerythrin-6A7 mouse monoclonal antibody to the GPA^M epitope and a fluorescein-BRIC157 mouse monoclonal antibody to the GPA^N epitope before high-speed (3000–4000 cells/s) flow cytometry on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). A total of 5×10^6 cells were analyzed for each sample using a rectangular gate of forward scatter *versus* log side scatter to discriminate against antibody-induced cell aggregates. GPA V_f were determined from the number of red cells falling within defined variant regions of the histogram divided by the total number of single cells analyzed.

Statistical Analysis of GPA V_f Data. The age distributions of patient, heterozygote, and control donors were compared using the nonparametric Mann-Whitney test. GPA V_f data are not normally distributed in most study or control populations. Thus, we calculated exponentiated means and standard deviations of log-transformed data as descriptive statistics and for significance testing. A two-sided *t* test on log-transformed data and the Mann-Whitney test were used to determine the statistical significance of V_f differences. Regression analyses were used to study the relationship between GPA V_f data and donor age. Because of the nonnormality of the data, nonparametric tests were used for inference regarding the slopes of V_f measures *versus* donor age. Specifically, the Spearman rank correlation test was used to test the null hypothesis of a zero slope for V_f *versus* age within a group (the hypothesis of no correlation is equivalent to the hypothesis of zero slope), and a permutation test was used to test for equality of the V_f-*versus*-age slopes between groups (22). Odds ratios and odds ratio confidence intervals and significance were calculated as previously described (Refs. 23 and 24, respectively). Statistical analyses were performed using S-Plus (MathSoft, Seattle, WA), DataDesk 5.0 (Data Description, Ithaca, NY), and StatView 4.5 (Abacus Concepts, Berkeley, CA).

RESULTS

Somatic Mutation in WRN Pedigrees. We analyzed GPA V_f in 11 WRN patients and 10 heterozygous family members who collectively carried 10 different *WRN* mutations (Table 1 and Fig. 2). The GPA Ø/N V_f was elevated in patients and in heterozygotes compared with matched control donors. In contrast, the N/N V_f of patients and heterozygotes did not differ from matched control donors (Fig. 3). The differences in mean GPA Ø/N V_f between WRN patients and controls, as well as between heterozygotes and controls, were highly significant ($P = 10^{-9}$ and $P \leq 0.0001$, respectively; Table 2). There was as much heterogeneity between patients with the same genotype as there was between patients with different *WRN* mutations (Table 1). WRN patients displayed higher median GPA Ø/N V_f than did heterozygotes, although this difference did not reach significance (Fig. 3, *left panel*; $P = 0.20$). There was no significant difference in N/N V_f between patients and controls ($P \leq 0.50$), between heterozygotes and controls ($P \leq 0.59$), or between patients and heterozygotes ($P = 0.50$; Table 2).

ORs calculated from comparing the frequency of individuals with high V_f in WRN patient and control populations were consistently significant at $V_f \geq 9 \times 10^{-6}$ (OR = 38.8, *i.e.*, the odds of being a WRN patient *versus* not being a patient are 38.8-fold higher at

Table 1 *GPA V_f and genotype of WRN patients and family members*

		GPA $V_f \times 10^{-6}$			
Sex	Age	Ø/N	N/N	WRN Pedigree[a]	Mutation(s)[a]
WRN$^-$/*WRN*$^-$					
F	21	7.2	10.0	SYR	Complex deletion
F	27	9.2	7.0	BLS	c.3724C→T
M	37	10.2	4.6	AUS	c.1396delA/IVS30+2T→A
M	39	31.8	10.0	DJG	c.1396delA/c.2320-3056del
M	41	13.2	5.0	CP6	IVS26+1G→C
F	42	20.0	6.4	CWW	c.3259-3262delCAAA/c.3265-3266delGA
F	47	37.1	59.2	CP2	c.1336C→T
M	47	22.8	7.4	MIKIT	IVS25−1G→C
F	48	14.2	9.4	LGS	c.1336C→T
M	56	52.9	21.8	JO	IVS25−1G→C
F	58	15.2	4.4	SANAN	c.2320-3056del
WRN$^+$/*WRN*$^-$					
M	14	9.8	2.6	TUR	c.3724C→T
M	20	6.8	4.2	TUR	c.3724C→T
F	21	7.6	5.0	SYR	Complex deletion
F	23	10.2	2.6	SYR	Complex deletion
M	29	19.2	7.8	SYR	Complex deletion
M	32	14.6	11.4	SYR	Complex deletion
M	48	6.0	11.0	SYR	Complex deletion
F	56	53.0	16.8	LGS	c.1336C→T
M	66	9.2	2.8	AUS	c.1396delA
M	72	18.0	51.6	SYR	Complex deletion
WRN$^+$/*WRN*$^+$					
M	15[b]	6.0	4.6	SYR	none

[a] *WRN* mutations and polymorphisms are detailed in the *WRN* Locus-Specific Mutational Database (URL: http://www.pathology.washington.edu/werner/; Ref. 5). The CWW and CP6 pedigree mutations are reported here for the first time.
[b] Estimated age.

Table 2 *GPA V_f in WRN patients and heterozygotes versus control donors*

GPA group and variant class	*n*	GPA $V_f \times 10^{-6}$				P^a	
		Range	Median	Geometric mean[b]	SD	*t* Test	Mann-Whitney
Ø/N							
WRN$^-$/*WRN*$^-$	11	7.2–52.9	15.2	17.7	9.5–33.0	10^{-9}	$<10^{-4}$
Controls	283	0.5–62.2	6.0	5.8	3.2–10.5		
WRN$^+$/*WRN*$^-$	10	6.0–53.0	10.0	12.3	6.4–23.4	10^{-4}	5×10^{-4}
Controls	362	0.4–211.4	6.0	6.1	3.1–11.8		
WRN$^-$/*WRN*$^-$	11	7.2–52.9	15.2	17.7	9.5–33.0	0.20	0.18
WRN$^+$/*WRN*$^-$	10	6.0–53.0	10.0	12.3	6.4–23.4		
N/N							
WRN$^-$/*WRN*$^-$	11	4.4–59.2	7.4	9.2	4.3–19.8	0.50	0.81
Controls	283	0.1–164.8	7.4	8.0	4.1–15.7		
WRN$^+$/*WRN*$^-$	10	2.6–51.6	6.4	7.1	2.7–18.7	0.59	0.43
Controls	362	0.0–246.6	7.6	8.1	3.7–17.5		
WRN$^-$/*WRN*$^-$	11	4.4–59.2	7.4	9.2	4.3–19.8	0.50	0.53
WRN$^+$/*WRN*$^-$	10	2.6–51.6	6.4	7.1	2.7–18.7		

[a] The statistical significance of GPA V_f differences are given as *P*s determined using a two-sided *t* test on log-transformed data and as a probability determined using the nonparametric Mann-Whitney test (19).

[b] GPA V_f data are not normally distributed (19). As a result, we have used geometric as opposed to arithmetic means and calculated SD as the exponentiated values of the log mean ± SD.

$V_f \geq 9 \times 10^{-6}$ than at $V_f < 9 \times 10^{-6}$). GPA $V_f \geq 9 \times 10^{-6}$ were observed in 10 of 11 (or 91%) of our WRN patients (Table 1). ORs calculated from N/N V_f data become significant at $V_f \geq 15 \times 10^{-6}$, although at a much lower OR of 3.5. Among *WRN* heterozygotes, Ø/N $V_f > 6 \times 10^{-6}$ were significant with ORs ≥ 10.8. These values included 9 of 10 (90%) of our *WRN* heterozygotes.

Age Regression Analyses. There was significant (nonzero) correlation between Ø/N V_f and age for both patient controls and heterozygote controls ($P = 0.02$ and $P < 0.0001$, respectively) as has been previously observed (for example, see Ref. 10). WRN patients demonstrated a steep increase in GPA Ø/N V_f with age compared with heterozygotes or controls (slope, 0.73 *versus* 0.25 and 0.09, respectively; Fig. 4), and this increase in patients differed significantly from controls ($P = 0.02$) as determined by permutation testing. Permutation testing was chosen as a relatively powerful nonparametric test because it requires no assumptions regarding the distribution of data in either the patient or control donor populations. Patients and heterozygotes did not have significantly different Ø/N V_f *versus* age slopes ($P = 0.38$), and neither did heterozygotes and controls ($P = 0.82$). N/N V_f was significantly correlated with age for patient controls ($P < 0.0001$) and for heterozygote controls ($P < 0.0001$). The N/N V_f age slopes for patients and heterozygotes did not differ from those for their respective control groups ($P = 0.60$ and $P = 0.23$, respectively), nor did they differ from each other ($P = 0.62$).

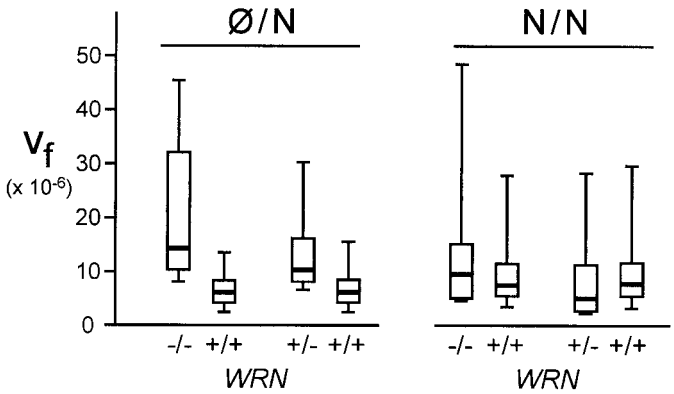

Fig. 3. GPA V_f as a function of *WRN* genotype. GPA N-allele V_f ($\times 10^{-6}$) are shown for WRN patients (*WRN*$^{-/-}$), heterozygous carriers (*WRN*$^{+/-}$), and matched control donors (*WRN*$^{+/+}$; see "Materials and Methods") as modified box plots. Boxes above each *WRN* genotype represent the central 50% of data points with the top and bottom of the box indicating 75th and 25th percentiles data, respectively. Median GPA N V_f for each *WRN* donor group are indicated by a horizontal line within the box, and the vertical lines extending from each box end indicate the 95th (top) and 5th (bottom) percentiles for V_f data for each donor group. Percentiles were determined directly for control donors and by interpolation for WRN patients and heterozygotes.

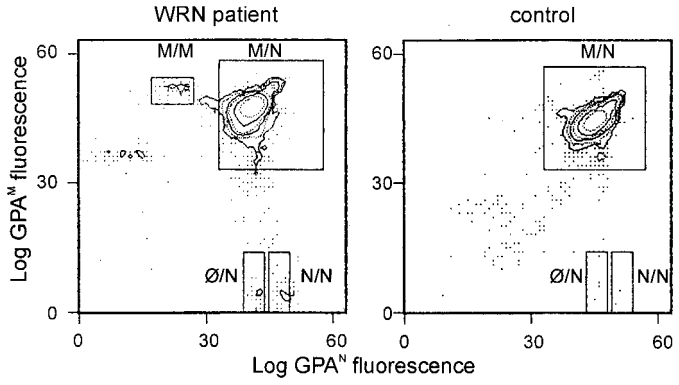

Fig. 2. Flow cytometric analysis of 10^6 erythrocytes from representative MN heterozygous Werner patient and control donor. Erythrocytes were immunolabeled for GPA M and N transmembrane proteins before flow cytometric analysis. The fluorescence intensity scales for GPA M (*Y* axis) and N (*X* axis) staining are given in log units. The major peak in each panel consists of M/N heterozygous cells. Rare Ø/N, N/N, and M/M-variant red cells are increased in the Werner patient (the 47-year-old female Werner patient listed in Table 1). Also noticeable in this patient is "tailing" between the M/N peak and the M/M and N allele variant boxes. This may arise due to somatic mutation during erythroid lineage expansion and differentiation.

DISCUSSION

We have used the GPA red cell V_f assay to quantify and characterize *in vivo* genetic instability in WRN patients and heterozygotes. WRN is an uncommon autosomal recessive disease in which progeroid features are associated with genetic instability and an elevated risk of neoplasia. Our study included 11 WRN patients and 10 within-pedigree heterozygotes who collectively carried 10 different *WRN* mutations. Three of these mutations, the 2-bp and 4-bp deletions identified in the CWW WRN pedigree and the CP6 splice-junction base subsitution, are reported for the first time (see Table 1). WRN patients and heterozygous family members had elevated GPA Ø/N V_f, indicating an elevated *in vivo* somatic mutation frequency.

The GPA Ø/N V_f elevations we observed in WRN patients are consistent with and corroborate our previous identification of an elevated mutant frequency in peripheral blood T lymphocytes from WRN patients (25) and a deletion mutator phenotype in WRN fibroblast cell lines (26). These three findings in concert indicate that the loss of WRN function promotes genetic instability in multiple human

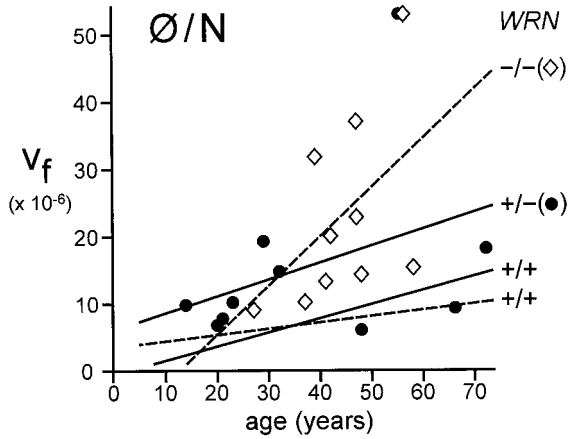

Fig. 4. GPA Ø/N variant frequency as a function of age and *WRN* genotype. The age regression of GPA Ø/N V_f ($\times 10^{-6}$) are shown for WRN patients ($WRN^{-/-}$) and heterozygous mutation carriers ($WRN^{+/-}$) together with matched control donors ($WRN^{+/+}$; see "Materials and Methods"). Patient (◇) and heterozygote (●) data points are shown together with corresponding regression line pairs for WRN patients and matched controls (*dashed line*) and for WRN heterozygotes and matched controls (*solid lines*). For clarity, data points for controls are not shown.

somatic cell lineages. The steep increase in Ø/N V_f as a function of patient age is particularly intriguing because it parallels the rapid rise in clinical signs, symptoms, and disease risk that begin in WRN patients after puberty (2, 27). This suggests that an increased mutation rate and/or mutation accumulation could be important in the WRN pathogenesis. The high OR we observed for Ø/N V_f in patients, of 38.8 at Ø/N $V_f \geq 9 \times 10^{-6}$ corresponding to a sensitivity of 91% and a specificity of 74%, indicates that the GPA assay may be useful in establishing a diagnosis of WRN in suspected patients. Although N/N V_f were not significantly elevated in WRN patients, Ø/N and N/N V_f covaried in patients at a level that approached significance (Pearson correlation = 0.6; $P = 0.07$). The only other patient population in which this type of Ø/N *versus* N/N V_f correlation has been observed is, intriguingly, BLM patients (9); BLM patients carry mutations in the *BLM* gene that encode a RecQ helicase related to WRN (18).

The identification of *in vivo* genetic instability in *WRN* heterozygotes was surprising and unanticipated. These results are, to the best of our knowledge, the first demonstration of genetic instability *in vivo* in heterozygotes for a human autosomal recessive genetic instability syndrome. Clinically important heterozygote effects have long been postulated for other of the recessive genetic instability syndromes, *e.g.,* ATM. *ATM* heterozygotes appear to be at increased risk for breast cancer and perhaps for other neoplasms. However, this increased risk is not clearly related to the modestly increased ionizing radiation sensitivity of *ATM* heterozygous cells, and there does not appear to be an excess of *ATM* heterozygotes among patients with a heightened or severe response to chemotherapy or ionizing radiation therapy (reviewed in Refs. 28–30). Moreover, no GPA heterozygote effect has been observed in ATM, BLM, or FAN pedigree analyses (9, 13, 15, 16).

The genetic instability we observed in *WRN* heterozygotes may have parallels in intermediate sensitivity of *WRN* heterozygous B lymphoblastoid cell lines to killing by 4-nitroquinoline 1-oxide (31) and by the DNA topoisomerase I inhibitor camptothecin[4] (32). The most likely mechanistic basis for these effects and for the genetic instability we observed *in vivo* in *WRN* heterozygotes is haplo-insufficiency. WRN protein and immune-precipitable WRN helicase activity are both reduced in cell lines from *WRN* heterozygotes. Moreover, the truncated WRN proteins predicted by mutant alleles are present at very low levels or are undetectable in patient and heterozygote cells (33, 34).

WRN heterozygotes appear to be relatively common in both the United States and Japan, with estimated frequencies of 1/200–1/500 (1, 35). Thus, a *WRN* heterozygote effect, if expressed as genetic instability or an enhanced sensitivity to DNA damaging agents, could be a predisposition to neoplasia or to adverse therapeutic effects. One way to test this hypothesis would be to look for an excess of mutant *WRN* alleles in neoplasms, especially of the types observed in Werner patients or in patients with adverse responses to drugs such as camptothecin that are selectively toxic to WRN cells (32, 36). Although the *WRN* open reading frame is large, mutation screening may be readily tractable because all of the known, clinically important *WRN* mutations thus far identified should be readily detected by an *in vitro* protein truncation screening assay (1, 5).

Our results also provide additional insight into the relationship between *in vivo* genetic instability and disease risk. ATM, BLM, and FAN patients display GPA V_f that are 10- to 100-fold higher than those of healthy controls and are at high risk of developing immunodeficiency, leukemia, lymphoma, and marrow failure (37, 38). Werner patients, in contrast, display more modest GPA V_f elevations and have a correspondingly lower risk of developing bone marrow neoplasia or failure (2, 3). This comparison suggests that a 10-fold or greater increase in somatic mutation confers a high risk of developing bone marrow and/or lymphoid pathology. The rapid increase in GPA Ø/N V_f with age in WRN patients predicts that older patients should be at higher risk of developing marrow pathology. This appears to be the case: leukemia, myelodysplasia, and myelofibrosis collectively represent 20% of nonepithelial neoplasms and 11% of all neoplasms in WRN patients (3, 39, 40). Moreover, patients who develop marrow dysfunction or neoplasia do so relatively late, with an average age of ~40 years at diagnosis (see Ref. 3 and additional references cited therein). Somatic mutation plays an important role in the genesis of at least one of the myelodysplastic syndromes, paroxysmal nocturnal hemoglobinuria (41–43), and may play a role in the propensity of the myelodysplastic syndromes to evolve into acute leukemias (44). Biochemical and functional analyses of WRN and the other human RecQ helicases should soon provide a better picture of *in vivo* RecQ helicase function and should indicate how the loss of function promotes genetic instability and the development of both neoplastic and non-neoplastic disease in specific human cell lineages (40, 45).

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