ORIGINAL ARTICLE

The Werner's syndrome 4330T>C (Cys1367Arg) gene variant does not affect the in vitro cytotoxicity of topoisomerase inhibitors and platinum compounds

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Abstract

Purpose Werner's syndrome (WS) is a recessive disorder of premature onset of processes associated with aging. Defective DNA repair has been reported after exposure of cells isolated from WS patients to DNA-damaging agents. The germline 4330T>C (Cys1367Arg) variant in the *WS* gene (*WRN*) has been associated with protection from age-related diseases, suggesting it has a functional role. We studied whether the 4330T>C variant confers altered drug sensitivity in vitro.

Methods 4330T>C was genotyped in 372 human lymphoblastoid cell lines (LCLs) from unrelated healthy Caucasian individuals using a TaqMan-based method. The study was powered to detect the effect of the 4330T>C genotypes after exposure to camptothecin (based upon preliminary data). The effect of the 4330T>C variant on the cytotoxicity of etoposide, carboplatin, cisplatin and daunorubicin was also tested. *WRN* expression in 57 LCLs was measured by microarray.

Results No significant difference between the IC50 of the cells was observed among genotypes ($P = 0.46$) after exposure to camptothecin. No association was also observed for etoposide, carboplatin, cisplatin, and daunorubicin (ANOVA, $P > 0.05$). *WRN* expression also did not vary across genotypes (ANOVA, $P = 0.37$).

Conclusion These results suggest that this nonsynonymous variant has relatively normal function at the cellular level.

Keywords Topoisomerase inhibitors ·
Werner's syndrome · Pharmacogenetics · Polymorphism ·
Cytotoxicity

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Abbreviations

WS	Werner's syndrome
WRN	Werner's syndrome gene
LCLs	Lymphoblastoid cell lines
4NQO	4-Nitro-quinoline-1-oxide
SNPs	Single nucleotide polymorphisms
EBV	Epstein–Barr virus

Introduction

Werner's syndrome (WS) is an autosomal recessive disorder characterized by premature onset of a number of

processes associated with aging. The major cause of death in individuals suffering from this disorder is cancer and myocardial infarction [5]. The WRN gene encodes a protein displaying helicase and exonuclease activities [33]. Moreover, the WS protein seems to be involved in the response to DNA damage during replication, as well as recombination and transcription processes (for a review on the function of the WS protein, refer to Ozgenc and Loeb [20]). Several mutations in the WRN gene are responsible for the occurrence of this syndrome, resulting in truncated gene products with loss of the C-terminal domain and precluding localization of the WS protein into the nucleus. This could represent the mechanistic basis to explain why most WS patients have similar clinical features even though they carry different mutations [16].

Defective DNA repair has been reported after exposure of cells isolated from WS patients to the genotoxic agent 4-nitro-quinoline-1-oxide (4NQO) and camptothecin [17, 18, 24]. Increased sensitivity to the topoisomerase I inhibitor camptothecin in cells derived from WS patients suggests that the WS protein functions primarily during DNA replication by correcting DNA lesions with high fidelity during the progression of the replication fork. In addition to topoisomerase I inhibitors, these cell lines are hypersensitive also to chromosomal damage induced by topoisomerase II inhibitors [23]. The WS protein cooperates with topoisomerase II, thus contributing to maintaining genomic integrity [7].

The 4330T>C (Cys1367Arg) variant is the most studied among the single nucleotide polymorphisms (SNPs) found in the WRN gene in subjects not affected by the syndrome. Previous epidemiologic studies suggest that this SNP plays a protective role against a variety of age-related disorders including risk of atherosclerosis and its complications [3, 32]. It has been speculated that, due to the presence of another basic amino acid (Arg coded by the variant allele) in the nuclear localization signal motif, this variant might enhance the translocation of the protein into the nucleus, allowing for more efficient activity of the WS protein in response to various challenges [4]. Moreover, it has been reported that B-lymphoblastoid cell lines (LCLs) having at least one copy of the mutated allele are more resistant to the cytotoxic effect induced by 4NQO, a genotoxic agent [17].

Instead of focusing on rare causative variants of WS that are unlikely to affect the outcome of a large number of cancer patients, we aimed to study the functional role of the germline 4330T>C variant. Contrary to the WS causative variants, this variant is common and might confer increased resistance to the effect of topoisomerase inhibitors and other DNA-damaging agents that are extensively used in the clinic to treat cancer patients. Hence, in order to investigate the effect of this variant on drug cytotoxicity, we performed a phenotype–genotype association study using LCLs treated with camptothecin, etoposide, and daunorubicin (topoisomerase inhibitors), as well as the DNA-damaging compounds carboplatin and cisplatin.

Materials and methods

Genotyping of the WRN 4330T>C variant

The 4330T>C variant was genotyped using a Taqman® pre-designed SNP genotyping assay (catalog number C___650486_10, Applied Biosystems, Foster City, CA). The reference sequence for the SNP is AF091214.1, and the dbSNP ID number is rs1346044. The Taqman probe-based PCR contained 2.5 μl of 2× Taqman Universal PCR Master Mix with Amperase UNG (P/N 4304437, Applied Biosystems), 0.25 μl of 20× Taqman SNP genotyping assay mix (including PCR primers and allele specific Taqman MGB probes, FAM and VIC dye-labeled) and 10 ng of genomic DNA in a total volume of 5 μl. The reactions were run at 50°C for 2 min in the beginning, followed by 10 min of denaturation at 95°C, and 40 cycles including 92°C for 15 s and 58°C for 1 min (ramp at 1°C/s). For fluorescence signal detection, the plate was read using a LJL Analyst AD instrument in the University of Chicago Genotyping Core. For pre-designed assays, Applied Biosystems does not provide the PCR primers and Taqman probe sequences. DNA samples with known genotype were used as controls.

Cytotoxicity of camptothecin

Out of the 372 genotyped cell lines (unrelated healthy Caucasians, 50% males) from the Coriell collection (Table S1), we selected 40 cell lines to use in the camptothecin cytotoxicity assays with TT ($n = 10$), CT ($n = 10$) and CC ($n = 20$) genotypes. The number of samples for each genotype was calculated based on preliminary data which indicated that we would have a 90% power to detect a 33% difference in IC_{50} for LCLs with CC genotype compared to those with the CT + TT genotypes with a sample of 40 LCLs (20 CC vs. 10 CT + 10 TT) [11].

Twenty-four hours after plating, media containing either vehicle (0.1% DMSO) or camptothecin (at 7 different concentrations ranging from 1–15 nM) were added to the 96-well plates containing exponentially growing LCLs (0.1 million cells/ml) and incubated for 72 h. Cytotoxicity was measured using the alamar blue assay (Biosource, Camarillo, CA) as described previously [9]. This is a rapid colorimetric assay where the reduction of alamar blue dye is proportional to the number of viable cells. Alamar blue

was added 24 h prior to reading of absorbances at wavelengths 570 and 600 nm using the Synergy-HT multidetection plate reader (BioTek, Winooski, VT). The absorbance readings were used to calculate % cell survival relative to the control:

$$\frac{(117,216 \times TestWellA570) - (80,586 \times TestWellA600)}{(117,216 \times ControlWellA570) - (80,586 \times ControlWellA600)} \times 100$$

Two separate experiments were performed in duplicate.

Cytotoxicity of etoposide, daunorubicin, carboplatin and cisplatin

The association between the 4330T>C genotypes and IC_{50} of 4 different drugs was tested in subsets of the 372 genotyped LCLs. The IC_{50} data were obtained from previous publications from our group [9] for etoposide (0, 0.02, 0.1, 0.5 and 2.5 μM; $n = 81$), daunorubicin (0, 0.0125, 0.025, 0.05, 0.1, 0.2 and 1.0 μM; $n = 82$), carboplatin (0, 10, 20, 40 and 80 μM; $n = 90$), and cisplatin (0, 1, 2.5, 5, 10 and 20 μM; $n = 63$). There was a high overlap ($n = 56$) in the cell lines used in these 4 cytotoxicity assays. At 24 h after plating, cells were treated with vehicle (0.01% DMSO for cisplatin, 0.05% for etoposide and 0.1% PBS for daunorubicin and carboplatin) or drug for 48 (cisplatin) or 72 h (etoposide, carboplatin and daunorubicin). Every experiment, with each concentration in triplicate, was performed in duplicate. Averaged viability in triplicate values (per concentration) from each experiment could not exceed more than a coefficient of variation (CV) of 0.15, otherwise the experiment was repeated.

Exon array gene-expression analysis

WRN mRNA expression was determined in LCLs by Affymetrix Exon Array 1.0 ST, as previously described [8]. There were 57 LCLs with both mRNA data and 4330T>C genotype information. Resulting probe-signal intensities were sketch-quantile normalized using a subset of the 1.4 million probe sets. Gene-expression levels were summarized using the robust multiarray average [12]. A constant of 16 was added for variance stabilization, and summarized signals were log2 transformed. This was performed using the signals generated on a core set of well-annotated refseq exons (>200,000) within the Affymetrix defined core set of >200,000 well-annotated exons. Probesets used to summarize *WRN* expression were mapped using Build 35 of the human genome. To prevent confounding interpretations of gene-expression variation, we removed data from exons for which probe sets contained two or more probes harboring SNPs before summarizing expression (GEO accession number GSE7761).

Sample size and power calculation

We calculated the number of LCLs needed to select 20 CC and 20 CT + TT LCLs for the camptothecin study. We relied on previous studies in Caucasians Trikka et al. [31] ($n = 80$) and Castro et al. [4] ($n = 352$)], where the frequency of the C allele was 0.31 and 0.30, respectively. Assuming no deviation from Hardy–Weinberg equilibrium, we expected the genotype frequencies to be 9, 42 and 49% for the CC, CT, and TT genotypes, respectively. As a comparison, in the larger study of Castro et al. [4], the genotype frequencies were 0.05, 0.43 and 0.52, respectively. Using 0.05 as the lowest expected frequency of the CC genotype, we estimated an overall sample size of 400 DNA samples. Hence, 232 DNAs from unrelated individuals of the CEPH families and 168 additional DNAs from unrelated Caucasian individuals were purchased from the Coriell collection for genotyping. All the LCLs used in this study are listed in Table S1, and are stratified by genotype.

Data analysis

Mean ± SD percent viability (linear value) was plotted against each corresponding concentration (log value). The concentration of drug required to inhibit 50% of cell viability (IC_{50}) was determined for each cell line by curve fitting of percent cell viability against all replicates at each concentration of drug. The curve was fit to the data using the following four parameters, Y_{max} = maximum responses, Y_{min} = minimum responses, $K_m = (Y_{max} + Y_{min})/2$, and n = slope. IC_{50} is determined by solving for concentration when Effect = 50%

$$\text{Effect} = \frac{Y_{max}K_m^n + Y_{min}\text{Conc}^n}{K_m^n + \text{Conc}^n} \quad (1)$$

Statistical significance was tested by t test of CC versus CT + TT (P cut-off of 0.05, one-tailed). The IC_{50} of camptothecin was normally distributed according to the Kolmogorov–Smirnov test (KS distance = 0.16, $P > 0.10$). For the other drugs, the distribution of the IC_{50} values of

etoposide, carboplatin, cisplatin and daunorubicin was not normal (KS distance = 0.45, $P < 0.0001$; KS distance = 0.17, P = 0.01; KS distance = 0.28, $P < 0.0001$; KS distance = 0.29, $P < 0.0001$, respectively). These data were log transformed prior to statistical analysis after which they passed the normality test (P > 0.10 for the 4 drugs). Statistical significance of the association between IC_{50} and the *WRN* variant was tested by ANOVA (P cut-off of 0.05). Pearson correlations were performed to determine if the cytotoxicity of daunorubicin, etoposide, carboplatin and cisplatin were related.

WRN mRNA expression levels were normally distributed (KS distance = 0.10 and P > 0.10) and the association between the *WRN* mRNA expression level and the *WRN* variant was tested by ANOVA ($P = 0.05$ for the cut-off, two-tailed).

Results

The 4330T>C allele frequency variant in 372 LCLs was 0.28, comparable to that previously reported in Caucasians [4, 31]. No deviation from Hardy–Weinberg equilibrium was observed ($P > 0.05$). The frequencies of the three genotypes were as follows: CC = 0.07, CT = 0.41, TT = 0.52.
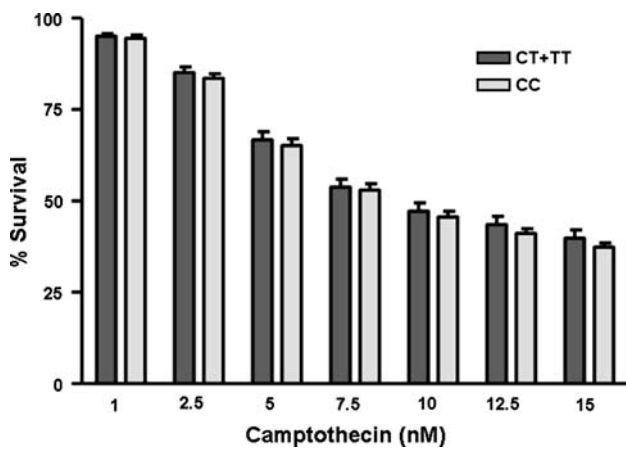
For the study of the effect of 4330T>C on camptothecin cytotoxicity, 40 LCLs (20 CC and 20 CT + TT, 50% males)

were randomly selected and exposed to a range of camptothecin concentrations for establishing the IC_{50}. Four cell lines (2 CT and 2 TT) that did not reach the IC_{50} and one not viable cell line (CC) were excluded. For the remaining 35 LCLs, the mean ± SD IC_{50} of camptothecin for the TT, CT, and CC genotypes were 9.0 ± 2.8 nM ($n = 8$), 8.2 ± 2.9 nM ($n = 8$) and 8.5 ± 2.1 nM ($n = 19$), respectively. These IC_{50} values are similar to those obtained by Okada et al. [18] in LCLs from normal individuals treated with camptothecin. There was no statistically significant difference between the IC_{50} in the CC group ($n = 19$, 8.5 ± 2.1 nM) compared to that of the CT + TT group ($n = 16$, 8.6 ± 2.8 nM) (one-tailed *t* test, $P = 0.46$; 95% confidence limits of the difference, −1.740 to 1.586 nM, Fig. 1a). Percentage viability at each camptothecin concentration did not differ among the three genotypes (for each concentration: one-tailed *t* test between CC and CT + TT, $P > 0.05$, Fig. 2). In addition, the IC_{50} for 4 LCLs required incubating them with higher camptothecin concentrations (up to 30 nM). The IC_{50} values of three of those LCLs were 13.0 (CT), 13.2 (TT), and 25.3 nM (TT). For one LCL (CT), the IC_{50} could not be estimated. As an exploratory objective, the IC_{50} values of these three LCLs were included in the analysis of the correlation between CC and CT + TT by using a rank test, and no significant difference was detected between CC vs. CT + TT [Mann–Whitney, one-tailed, $P = 0.26$, median (range) of 8.7 nM (5.0–12.3) vs. 7.8 nM (5.2–25.3), respectively)].
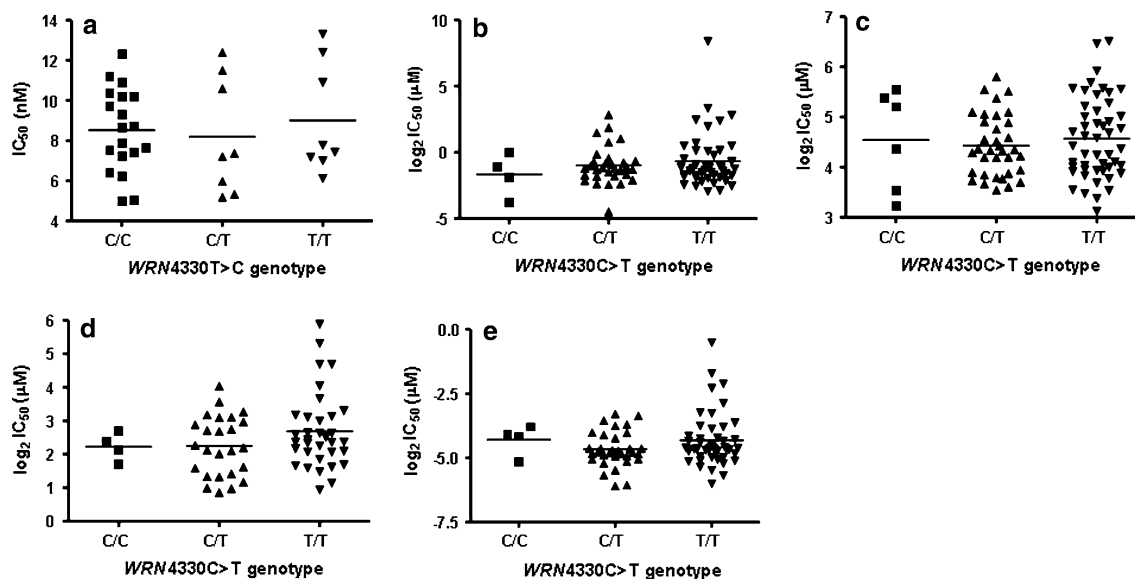

Fig. 1 Cytotoxicity in LCLs treated with **a** camptothecin, **b** etoposide, **c** carboplatin, **d** cisplatin and **e** daunorubicin stratified by the 4330T>C genotype The IC_{50} data for etoposide (0, 0.02, 0.1, 0.5 and 2.5 μM; $n = 81$), daunorubicin (0, 0.0125, 0.025, 0.05, 0.1, 0.2 and 1.0 μM; $n = 82$), carboplatin (0, 10, 20, 40 and 80 μM; $n = 90$), and cisplatin (0, 1, 2.5, 5, 10 and 20 μM; $n = 63$) were obtained from previous publications from our group [19]. At 24 h after plating, cells were treated for 48 h with cisplatin ($n = 3$ for each concentration) or 72 h with camptothecin ($n = 2$ for each concentration), etoposide, carboplatin, daunorubicin ($n = 3$ for each concentration). Cytotoxicity was measured using the alamar blue assay. All experiments were performed in duplicate. *Bars* represent mean values

Fig. 2 Percentage cell viability of LCLs with CC, CT, and TT genotypes at each camptothecin concentration. Data are expressed as mean $\pm$ SD

For the study of the effect of 4330T>C on the cytotoxicity of daunorubicin, etoposide, cisplatin and carboplatin, no differences were observed between the IC_{50} values across the 4330T>C three genotypes for any of the drugs tested (ANOVA, $P = 0.50$ for etoposide (Fig. 1b), $P = 0.69$ for carboplatin (Fig. 1c), $P = 0.24$ for cisplatin (Fig. 1d) and $P = 0.24$ for daunorubicin (Fig. 1e). No association could be detected when WRN expression was stratified by genotype (ANOVA, $P = 0.37$, Fig. 3). No significant association was observed between the levels of WRN mRNA expression in LCLs and the IC_{50} of etoposide, daunorubicin, carboplatin and cisplatin (data not shown). The cytotoxicities of the following drugs were related: (1) carboplatin and cisplatin (Pearson $r = 0.53$, $P < 0.0001$, $n = 63$), (2) etoposide and cisplatin ($r = 0.50$, $P < 0.0001$, $n = 57$), (3) daunorubicin and etoposide ($r = 0.46$, $P < 0.0001$, $n = 74$), (4) etoposide and carboplatin
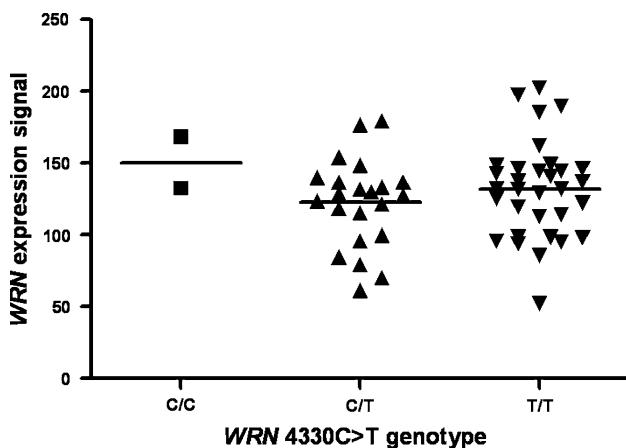
Fig. 3 WRN mRNA expression data according to the 4330T>C genotype. *Bars* represent mean values. *Y*-axis represents transcript level expression measured using the Affymetrix HuEx 1.0 ST Array ($n = 57$), as described under "Materials and methods"

($r = 0.41$, $P = 0.0002$, $n = 80$) and (5) daunorubicin and cisplatin ($r = 0.33$, $P = 0.01$, $n = 55$). However, the cytotoxicities of daunorubicin and carboplatin were not related ($r = 0.17$, $P = 0.12$, $n = 81$).

Discussion

This study demonstrates that the 4330T>C (Cys1367Arg) polymorphism in the WRN gene has no apparent effect on the sensitivity of LCLs to camptothecin, a prototype topoisomerase inhibitor used in several studies of the interaction between the WS protein and cellular topoisomerase I activity [10, 15, 19, 22, 27, 31]. In addition, this variant does not seem to have an effect on the sensitivity to other drugs including topoisomerase II inhibitors and platinum DNA-damaging agents.

This polymorphism does not result in a truncated WRN protein, and is close to the nuclear localization signal motif. It has been speculated that the presence of another basic amino acid (Arg coded by the variant allele) in the nuclear localization signal motif might enhance the strength of the nuclear localization signal, providing the potential for a more rapid and efficient transport of the protein to the nucleus in response to various challenges [4]. In addition, Castro et al. [4] reported (as data not shown) that LCLs having at least one copy of the mutated allele are more resistant to the cytotoxic effect induced by 4NQO, a genotoxic agent frequently used to induce DNA damage in WS-defective LCLs collected from WS patients [17]. These results are not confirmed by our data, as the LCLs with the CC genotype were equally sensitive to those with either the CT or the TT genotype when exposed to camptothecin.

At the time of the design of this study, the molecular function of this variant was not known. However, Kamath-Loeb et al. (2004) [13] demonstrated that the helicase/exonuclease activity of the mutated protein is unchanged, as well as its expression level, in agreement with our microarray data (Fig. 3). Further evidence of the lack of function of the 4330T>C variant comes from the studies of Bohr et al. [2], where both the helicase/nuclease activity of the Arg-containing protein did not differ from that of the Cys-containing protein. These studies also ruled out an effect on protein nuclear translocation and localization [2]. Our studies and the accumulated evidence of the lack of molecular effects of the 4330T>C variant strongly suggests that this variant is unlikely to alter the DNA-repair activity of the WS protein in cellular systems.

Although it has been demonstrated that WS cells are hypersensitive to chromosomal damage induced by etoposide [23], we could not detect any effect of 4330T>C on the cytotoxicity of the topoisomerase II inhibitors

etoposide and daunorubicin, in line with the results obtained with camptothecin. With regard to the lack of effect of 4330T>C on the cytotoxicity of platinum compounds, very recent findings [6] indicate that the sensitivity to cisplatin is only marginally reduced in WS$^{-/-}$ DT40 cells, suggesting a minor role of the WS protein on repairing platinum-induced DNA damage. The extent of DNA damage of platinum compounds is rather dependent upon the correct activity of other DNA repair proteins, including proteins of the nucleotide excision repair system and the related XPA and BRCA1 (Stewart [30]; Rabik and Dolan [25]). There was a high overlap in the cell lines used in the cytotoxicity assays. However, we acknowledge that by not using exactly the same cell lines for the different drug cytotoxicity screening assays, we have introduced increased heterogeneity into the analyses as each cell line would have a unique genotype for the proteins involved in the DNA base excision repair pathway.

The protection from age-related diseases conferred by the Arg variant initially reported by Castro et al. (2000) [3] and Ye et al. [32] were not subsequently confirmed by others. For example, several studies in the elderly have not found an association with coronary artery disease [2], Alzheimer's disease [21], age-related morbidities [29], and aging-trajectories and survival [14]. In addition, several WRN gene variants have been associated with the myelotoxic effects on benzene exposure in workers, but the 4330T>C variant showed no effect [28]. It seems evident that the complete deficiency of the WS protein predisposes WS patients to the risk of suffering severe side effects of chemotherapy, as suggested by a toxic death of a WS patient who developed acute myelogenous leukemia and was treated with standard doses of cytarabine, mitoxantrone and etoposide [26]. Similarly, epigenetic inhibition of the WS protein through hypermethylation in colorectal tumors increased the survival of patients treated with irinotecan, the most commonly used topoisomerase I inhibitor in the clinic [1]. These studies indicate that the pharmacodynamics of topoisomerase inhibitors might be affected when the activity of the WS protein is dramatically reduced. However, it remains to be investigated in a clinical setting whether the 4330T>C or other WRN germline variants that have more subtle molecular effects might increase the incidence of myelotoxicity of topoisomerase inhibitors and DNA-damaging agents. Functional validation of common WRN germline variation should be conducted before testing the role of WRN variants in a clinical setting. Additional studies should be conducted at haplotype level to understand the interaction among SNPs in the WRN gene.

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