Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer

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Werner syndrome (WS) is an inherited disorder characterized by premature onset of aging, genomic instability, and increased cancer incidence. The disease is caused by loss of function mutations of the WRN gene, a RecQ family member with both helicase and exonuclease activities. However, despite its putative tumor-suppressor function, little is known about the contribution of WRN to human sporadic malignancies. Here, we report that WRN function is abrogated in human cancer cells by transcriptional silencing associated with CpG island-promoter hypermethylation. We also show that, at the biochemical and cellular levels, the epigenetic inactivation of WRN leads to the loss of WRN-associated exonuclease activity and increased chromosomal instability and apoptosis induced by topoisomerase inhibitors. The described phenotype is reversed by the use of a DNA-demethylating agent or by the reintroduction of WRN into cancer cells displaying methylation-dependent silencing of WRN. Furthermore, the restoration of WRN expression induces tumor-suppressor-like features, such as reduced colony formation density and inhibition of tumor growth in nude mouse xenograft models. Screening a large collection of human primary tumors ($n = 630$) from different cell types revealed that WRN CpG island hypermethylation was a common event in epithelial and mesenchymal tumorigenesis. Most importantly, WRN hypermethylation in colorectal tumors was a predictor of good clinical response to the camptothecin analogue irinotecan, a topoisomerase inhibitor commonly used in the clinical setting for the treatment of this tumor type. These findings highlight the importance of WRN epigenetic inactivation in human cancer, leading to enhanced chromosomal instability and hypersensitivity to chemotherapeutic drugs.

DNA methylation

Werner syndrome (WS) is an autosomal recessive disease characterized by premature aging and a high incidence of malignant neoplasms (1, 2). Mutations in the WS gene (WRN) are found in patients exhibiting the clinical symptoms of WS (3–5). The vast majority of WRN mutations result in loss of function of the WRN protein (6). The WRN protein has been demonstrated to possess helicase and exonuclease activities (7–9), and cultures of WS cells show increased chromosomal instability, with abundant deletions, reciprocal translocations, and inversions (10, 11).

WRN belong to the RecQ family of helicases, which are highly conserved from bacteria to human, and whose members are thought to be essential caretakers of the genome (11, 12). In addition to WRN, germline mutations of two other RecQ helicases, BLM in Bloom syndrome and RECQL4 in Rothmund–Thomson syndrome, are also associated with an elevated incidence of cancer (12). Because patients with WRN germline mutations develop a broad spectrum of epithelial and mesenchymal tumors, which is one of the main causes of their death before the age of 50, a tumor-suppressor function for WRN has been proposed. This putative role is also supported by a very high rate of loss of heterozygosity at the chromosomal WRN loci at 8p11.2–p12 in many tumor types, including colorectal and breast cancer (13, 14). However, somatic mutations of WRN have not been described in sporadic neoplasms. Transcriptional inactivation by CpG island promoter hypermethylation is an alternative and emergent mechanism for the inactivation of tumor-suppressor genes (15–18). Similar scenarios to that outlined for WRN have been described for other DNA-repair familial-tumor-suppressor genes, such as hMLH1 and BRCA1, which are very rarely mutated in sporadic tumors but undergo epigenetic inactivation by hypermethylation of their regulatory regions in noninherited neoplasms (15, 17, 19, 20).

In this manuscript, we demonstrate that WRN undergoes CpG island promoter methylation-associated gene silencing in human cancer cells. The hypermethylation of the WRN promoter leads to its loss of expression and hypersensitivity to topoisomerase inhibitors and DNA-damaging agents. The epigenetic loss of WRN function can be rescued by the use of DNA-demethylating agents. Furthermore, the reintroduction of WRN into those transformed cell lines with WRN-deficiency due to hypermethylation provokes a reduction in colony formation and a decrease in growth of tumor xenografts, supporting the hypothesis that WRN has a tumor-suppressor role. The analysis of a large panel of human primary tumors ($n = 630$) shows that WRN CpG island hypermethylation is a common event in tumorigenesis. Most importantly, for colorectal cancer, the presence of aberrant methylation at the WRN promoter predicts improved survival in those patients treated with irinotecan, a topoisomerase inhibitor commonly used in this neoplasm. These findings underline the significance of WRN as a caretaker of our genome with tumor-suppressor activity and identify epigenetic silencing of WRN as a key step in cancer development that may have an important clinical influence on the treatment of these patients.

Results

WRN Promoter CpG Island Hypermethylation Leads to Gene Inactivation. WRN is a gene candidate for hypermethylation-associated inactivation in human cancer because a 5′-CpG island is located around the transcription start site (Fig. 1*A*). To analyze the methylation status of the promoter-associated CpG island, we screened seven human cancer cell lines from three different cell types of malignancies, colon (HCT-116 and COLO-205), breast (MCF-7 and MDA-MB-231), and leukemia (HL-60, U937, and REH), using bisulfite genomic sequencing and methylation-specific PCR targeted to the area surrounding the transcription start site, as

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Abbreviations: siRNA, small interfering RNA; WS, Werner syndrome.

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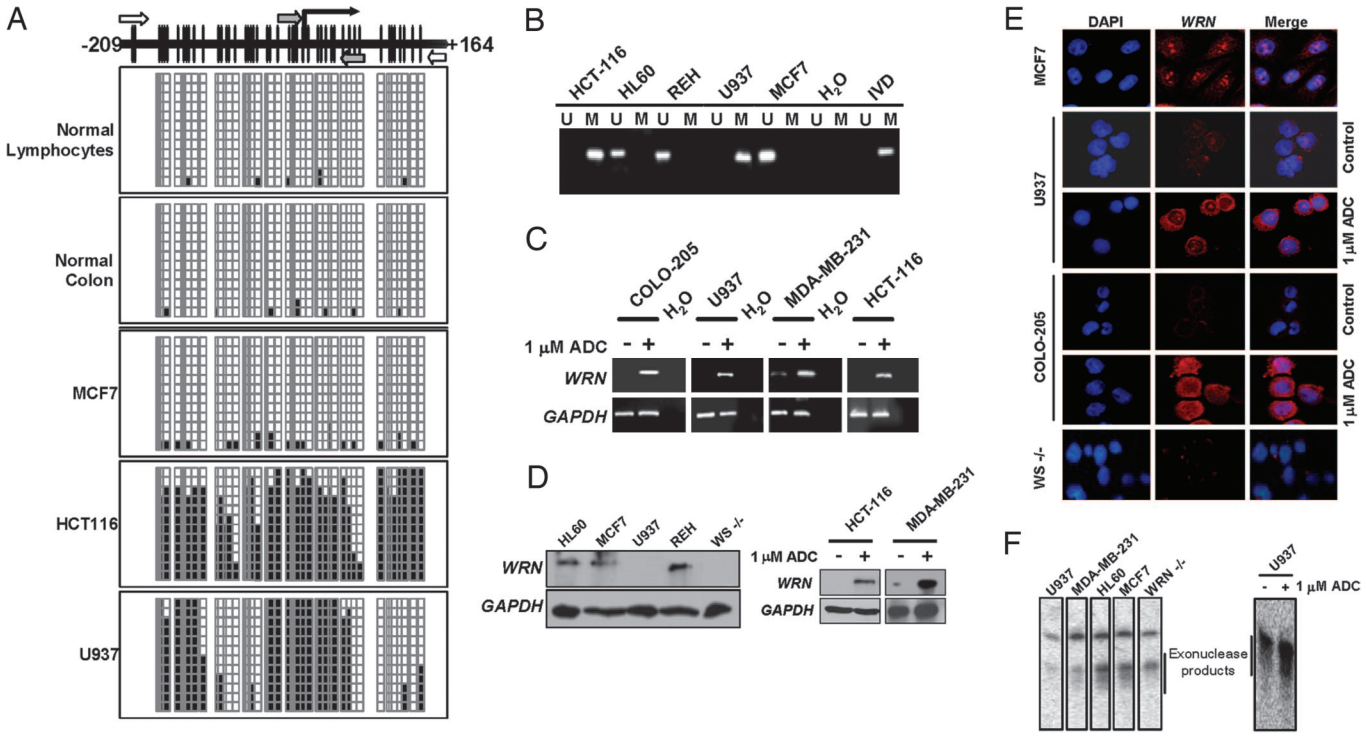

Fig. 1. Analysis of WRN CpG island promoter methylation status and gene function in human cancer cell lines. (*A*) Schematic depiction of the WRN CpG island around the transcription start site (long black arrow). CpG dinucleotides are represented as short vertical lines. Location of bisulfite genomic sequencing PCR primers and methylation-specific PCR primers are indicated as white and gray arrows, respectively. Shown are results of bisulfite genomic sequencing of 12 individual clones. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. (*B*) Methylation-specific PCR for the WRN gene in human cancer cell lines. The presence of a PCR band under lanes M or U indicates methylated or unmethylated genes, respectively. *In vitro* methylated DNA (IVD) is used as positive control for methylated DNA. (*C*) RT-PCR analysis of WRN expression. Treatment with the demethylating agent (ADC + lanes) reactivates WRN gene expression. (*D*) Western blot analysis of WRN expression. The WRN hypermethylated cell lines HCT-116, MDA-MB-231, and U937 do not express the WRN protein or have minimal expression (MDA-MB-231). The treatment with the demethylating agent reactivates WRN gene expression. WS−/− cells are shown as negative control. (*E*) Immunofluorescence analysis of WRN expression. The methylated cell lines COLO-205 and U937 and the mutant WS−/− cells do not stain for the WRN protein, in comparison with the unmethylated MCF-7 cells. Treatment with the demethylating agent (DAC) restores protein expression. (*F*) Exonuclease activity assay in WRN-immunoprecipitated cell resolved on denaturing polyacrylamide gels. The 3′-recessed duplex substrate used for exonuclease studies was degraded more extensively in WRN unmethylated cells (MCF-7 and HL60) than in WRN methylated (U937 and MDA-MB-231) or mutated (WRN−/−) cells.

described in *Methods*. WRN CpG island promoter hypermethylation was found in four cancer cell lines: HCT-116, COLO-205, MDA-MB-231, and U937 (Figs. 1 *A* and *B*). All normal tissues analyzed, including lymphocytes, bone marrow, breast, colon, and skin, were completely unmethylated at the WRN promoter (Fig. 1*A*).

Having noted WRN promoter hypermethylation in cancer cell lines, we assessed the association between this epigenetic aberration and the putative transcriptional inactivation of the WRN gene at the RNA and protein levels. The cancer cell lines HCT-116, COLO-205, MDA-MB-231, and U937 hypermethylated at the WRN CpG island did not express (HCT-116, COLO-205, and U937) or had minimal expression of (MDA-MB-231) the WRN RNA transcript, as determined by RT-PCR (Fig. 1*C*) and WRN protein, as determined by Western blot (Fig. 1*C*) and immunofluorescence (Fig. 1*E*). In contrast, MCF-7, HL-60, and REH, unmethylated at the WRN promoter, expressed WRN protein (Fig. 1 *C–E*).

We established a further link between WRN CpG island hypermethylation and its gene silencing by the treatment of the methylated cell lines with a DNA-demethylating agent. The treatment of the HCT-116, COLO-205, MDA-MB-231, and U937 cell lines with the demethylating drug 5-aza-2′-deoxycytidine restored the expression of WRN RNA transcript and protein (Fig. 1 *C–E*). It is not only a matter of restoring gene expression but also of rescuing gene functionality. This is exemplified by several genes that undergo methylation-associated silencing, such as the DNA-repair gene hMLH1, the MDM2-regulator $p14^{ARF}$, or the glycosyltransferase EXT-1, where treatment with the demethylating agent induced recovery of gene functions: DNA mismatch repair activity, sequestration of MDM2, and heparan sulfate biosynthesis, respectively (19, 21, 22). Because WRN is the only RecQ member that exhibits exonuclease activity (9), we examined the impact of WRN methylation-mediated silencing in this enzymatic function of WRN and the effect of restoring WRN expression by pharmacological means. We observed a loss of exonuclease enzymatic activity in WRN-immunoprecipitated cell extracts of U937 and MDA-MB-231, all of them hypermethylated at the WRN promoter, compared with HL60 and MCF-7 cells, these latter two having an unmethylated WRN promoter (Fig. 1*F*). Most important, treatment of U937 cells with the demethylating agent 5-aza-2′-deoxycytidine induced a significant increase in the WRN-associated exonuclease activity in these cells (Fig. 1*F*).

Reintroduction of WRN in Hypermethylation-Deficient Cancer Cell Lines Has Tumor-Suppressor-Like Properties. Although it has been proposed before that WRN has tumor-suppressor gene features, we assayed the ability of WRN to function as a suppressor of tumor growth in our model, using the breast cancer cell line MDA-MB-231 with WRN methylation-associated silencing. We first tested the inhibitory abilities of WRN in a colony-focus assay using G418 selection after transfection with the WRN gene (pEGFP-C1-

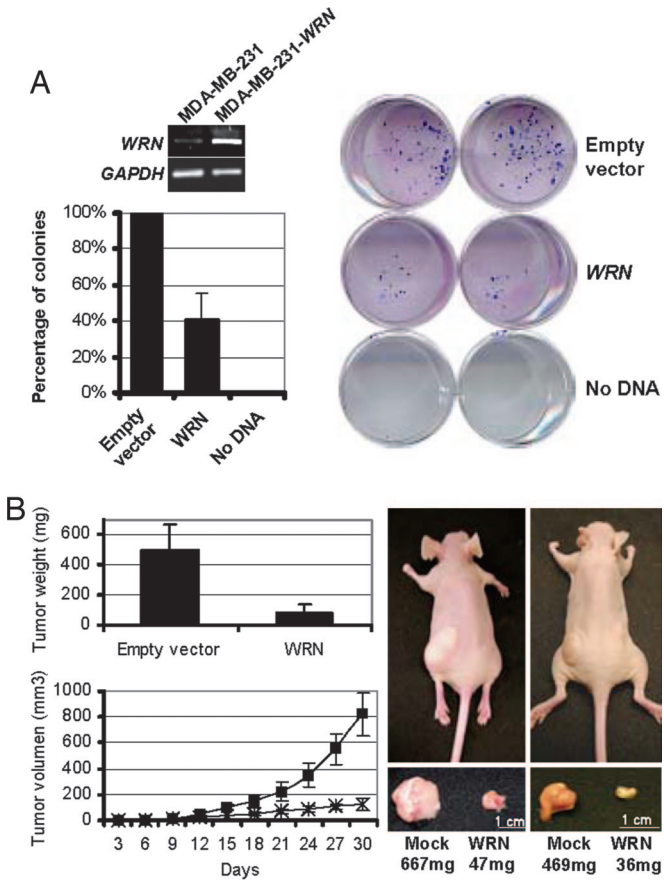

Fig. 2. Tumor-suppressor-like properties of WRN reintroduction. (*A*) Colony-formation assay. (*Upper Left*) WRN expression monitored by RT-PCR in untransfected and WRN-transfected MDA-MB-231 cells monitored by RT-PCR. (*Lower Left*) Densitometric quantification of the colony formation density of MDA-MB-231 cells transfected with the empty vector or with WRN. Three independent experiments were carried out. (*Right*) Example of the colony focus assay after a 2-week selection with G418 and staining with methylene blue. (*B*) Effect of WRN transfection on the *in vivo* growth of MDA-MB-231 cells. Tumor weight and size was monitored over time. Shown are female athymic nude mice 30 days after injection of 10^6 MDA-MB-231 cells. Note the large tumor on the left flank, corresponding to empty vector MDA-MB-231 cells, and the small tumor on the opposite flank, corresponding to WRN-MDA-MB-231 cell injection.

WRN) or the empty vector (pEGFP-C1). WRN expression was monitored by RT-PCR (Fig. 2*A*). Inhibition of tumor-cell growth was assessed by seeding MDA-MB-231-transfected cells on methylcellulose, incubating for 15 days, and then scoring the number of colonies formed. WRN re-expression demonstrated tumor-suppressing activity with a marked reduction of 59 ± 10% in colony-formation density with respect to the empty vector (Fig. 2*A*).

We next tested the ability of WRN-transfected MDA-MB-231 cells to form tumors in nude mice compared with that of empty-vector-transfected MDA-MB-231 cells. The same mice were s.c. injected with 10^6 WRN or empty-vector-transfected MDA-MB-231 cells (Fig. 2*B*). All mice were killed 30 days after the injection, and the tumors were dissected and weighed. Cells transfected with the empty vector (MDA-MB-231/pEGFP-C1) formed tumors rapidly, but cells infected with the WRN expression vector (MDA-MB-231/pEGFP-C1-WRN) had much lower tumorigenicity (Fig. 2*B*). At the time of death, tumors were six times larger in those mice with the empty vector, 500 ± 167.3 mg, than in those xenografts arising in mice transfected with WRN, 86 ± 50.4 mg (Fig. 2*B*).

Hypermethylation-Deficient WRN Cancer Cells Are Sensitive to Inhibitors of Topoisomerase I and DNA-Damaging Agents. It is already well known that lymphoblastoid cells and fibroblasts established from WS patients (11, 23–26) and embryonic stem cells from WRN-deficient mice (27) are hypersensitive to chromosomal damage and apoptosis upon their exposure to topoisomerase inhibitors and DNA cross-linking drugs. Related to the mechanism of apoptosis upon exposure to topoisomerase inhibitors, it has been associated with the persistence of a topoisomerase I–DNA intermediate in WRN−/− cells from WRN patients that it generates stalled replication forks (28). It would be extremely interesting to know, for clinical translational purposes, whether cancer cells with WRN-methylation-associated silencing also display these functional features. To address this issue, we treated WRN-unmethylated and hypermethylated cancer cell lines with camptothecin (a topoisomerase I inhibitor) or mitomycin C (an interstrand crosslinker) and measured the apoptotic rate by flow cytometry and chromosomal breakage by cytogenetic analysis.

For the apoptosis study, we observed that both camptothecin and mitomycin C were optimal inductors of apoptosis in the breast cancer cell line MDA-MB-231 and the leukemia cell line U937 (Fig. 3*A*; and see Fig. 5, which is published as supporting information on the PNAS web site), both of which display WRN promoter hypermethylation. The same phenomenon was observed for the cell line from a WS patient used as a positive control (Fig. 3*A*). By striking contrast, the breast cancer cell line MCF-7, unmethylated at the WRN promoter, was markedly resistant to camptothecin and mitomycin C-induced apoptosis (Fig. 3*A*). Most importantly, when we used WRN-transfected U937 and MDA-MB-231 cells, the resulting cells became more resistant to the apoptosis mediated by both drugs (Figs. 3*A* and 5).

The results of the chromosomal breakage analysis reflect those obtained from the apoptotic assays. Human cancer cell lines with an unmethylated WRN promoter, such as the breast cancer MCF-7 or the leukemic HL60 cells, demonstrated minimal chromosomal breakage upon exposure to mitomycin (Fig. 3*B*). In contrast, both cells from a WS patient and the breast MDA-MB-231 cells with WRN aberrant methylation were extremely sensitive to the drug, and a high frequency of chromosomal breakages, sometimes even in the characteristic form of quatriradial chromosomes, were observed in metaphases (Fig. 3*B*). Furthermore, when WRN-transfected MDA-MB-231 cells were exposed to mitomycin C, they acquired resistance to chromosomal breakage (Fig. 3*B*). Finally, the down-regulation of WRN by RNA interference in cells with an unmethylated promoter (MCF-7) was associated with an increased ratio of chromosomal breakage upon exposure to mitomycin C (Fig. 3*C*). All of these results establish a tight link between loss of WRN and increased sensitivity to the chemotherapeutic drug.

Profile of WRN CpG Island Hypermethylation in Human Primary Malignancies. After the demonstration of the epigenetic loss of function of WRN in cancer cell lines, we assessed the prevalence of WRN CpG island promoter hypermethylation in cancer patients. We examined 630 primary tumors corresponding to 11 different tissue types. WRN CpG island promoter hypermethylation was observed at different frequencies in each class of neoplasm and was present in epithelial and mesenchymal tumors (Fig. 4*A*). WRN hypermethylation of epithelial tumors was most prevalent in colorectal cancer (37.9%, 69/182), followed by non-small cell lung (37.5%, 21/56), gastric (25%, 10/38), prostate (20%, 4/20), breast (17.2%, 10/58), and thyroid (12.5%, 4/32) tumors. Of the hematological malignancies, promoter methylation of the WRN gene was often found in non-Hodgkin lymphoma (23.7%, 28/118) but was much less common in acute lymphoblastic leukemia (9.5%, 2/21) and acute myeloblastic leukemia (4.8%, 3/63). For the mesenchymal tumor types, WRN hypermethylation was present in chondrosarcomas (33.3%, 5/15) and osteosarcomas (11.1%, 3/27).

To further confirm the correlation between WRN hypermeth-

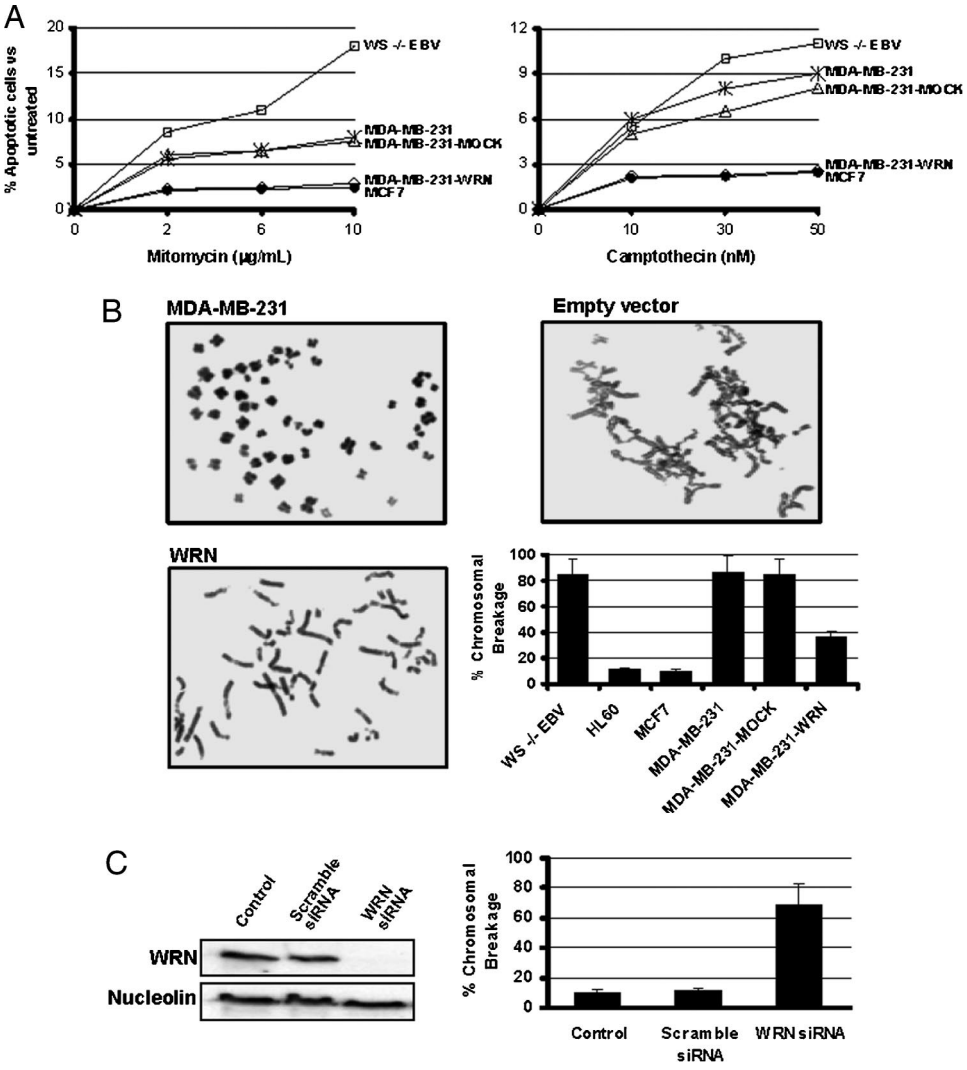

Fig. 3. Hypermethylation-deficient WRN cancer cells are sensitive to inhibitors of topoisomerase I and DNA-damaging agents. (*A*) Induction of apoptosis measured by flow cytometry in unmethylated (MCF-7), methylated (MDA-MB-231), and mutated (WS−/−) WRN cells at increasing concentrations of camptothecin and mitomycin C. MDA-MB-231 and WS−/− cells are highly sensitive in comparison with MCF-7. Restoration of WRN expression in MDA-MB-231 cells induces resistance to apoptosis by both drugs. (*B*) Chromosomal breakage measured by cytogenetic analysis of metaphase chromosomes. (*Upper Left*) Untreated MDA-MB-231 cells have undetectable fragility. (*Upper Right*) 50 mg/ml mitomycin C-treated cells undergo a massive breakage (empty vector). (*Lower Left*) MDA-MB-231 cells transfected with the WRN gene display resistance to the genome damage. (*Lower Right*) Quantification of chromosomal breakage induced by mitomycin C in cells proficient (HL60 and MCF-7) or deficient in WRN function by mutation (WS−/−) or methylation (MDA-MB-231). Transfection of the WRN gene in MDA-MB-231 cells provokes resistance to the genomic damage induced by the drug. (*C*) siRNA assay for the WRN transcript in MCF-7 cells. (*Right*) Western blot of WRN-knocked-down MCF-7 cells by siRNA. (*Left*) Quantification of chromosomal breakage induced by mitomycin C in MCF-7 proficient cells or cells deficient in WRN function by siRNA. WRN-knocked-down MCF-7 cells are prone to chromosomal breakage upon exposure to mitomycin C.

ylation and loss of WRN protein also in primary tumors, we conducted a double-blind analysis in primary gastric and colorectal tumors. For 16 cases of gastric tumors, an immunohistochemistry study of the WRN protein was carried out. In all cases with an unmethylated WRN promoter ($n = 8$) the WRN protein was strongly expressed; in contrast, the 8 methylated cases lacked WRN expression (Fig. 4*B*). We also analyzed 16 cases of colorectal tumors using WRN Western blotting. We found again that all of the unmethylated cases ($n = 8$) expressed the WRN protein, whereas for the methylated cases ($n = 8$), 7 (87%) lacked WRN expression and only 1 remaining case retained minimal expression of the WRN protein (Fig. 4*B*).

WRN Hypermethylation in Colon Cancer Patients Predicts Good Response to the Camptothecin Analogue Irinotecan. Because cell lines from WS patients are extremely sensitive to the drug camptothecin (11, 23–26), and given our finding, above, that human cancer cells with WRN-methylation-associated silencing are also very sensitive to this drug, we wondered whether these observations could also be translated to clinical samples. In this context, one camptothecin analogue, irinotecan (CPT-11), has been approved for clinical use in the treatment of colon cancer, a tumor type in which we found a significant rate of WRN CpG island hypermethylation.

We therefore assessed whether the presence of WRN promoter hypermethylation was a predictive marker of response to irinotecan in colorectal cancer patients treated with this drug. We selected a similar number of WRN-hypermethylated ($n = 45$) and unmethylated ($n = 43$) primary colorectal tumors from patients treated with irinotecan for whom a long clinical follow-up was available. We found that the median time for death of patients was 39.4 months for WRN methylated colon tumors but only 20.7 months for WRN unmethylated colon tumors. Thus, the presence of WRN CpG island promoter hypermethylation was a significant predictor of increased overall survival in colon cancer patients treated with irinotecan (Kaplan–Meier, $P = 0.00005$; 95% confidence interval, 25.4–35.2) (Fig. 4*C*).

Discussion

Aging is the main risk factor associated with cancer development (29). Thus, it makes sense that the inactivation of a gene involved in "preventing" the aging process occurs in cancer cells. We recently found the first example of this concept: lamin A/C (LMNA) is mutated in atypical WS, where the WRN gene is wild type (30), whereas LMNA undergoes methylation-associated silencing in hematological neoplasms (31). Here, we demonstrate that the first and paradigmatic premature aging gene, WRN, undergoes epigenetic inactivation in human cancer and can be viewed as a tumor-suppressor gene.

Patients with WS display a remarkable number of clinical signs and symptoms associated with premature aging, including graying of the hair, cataracts, osteoporosis, diabetes, and atheroscle-

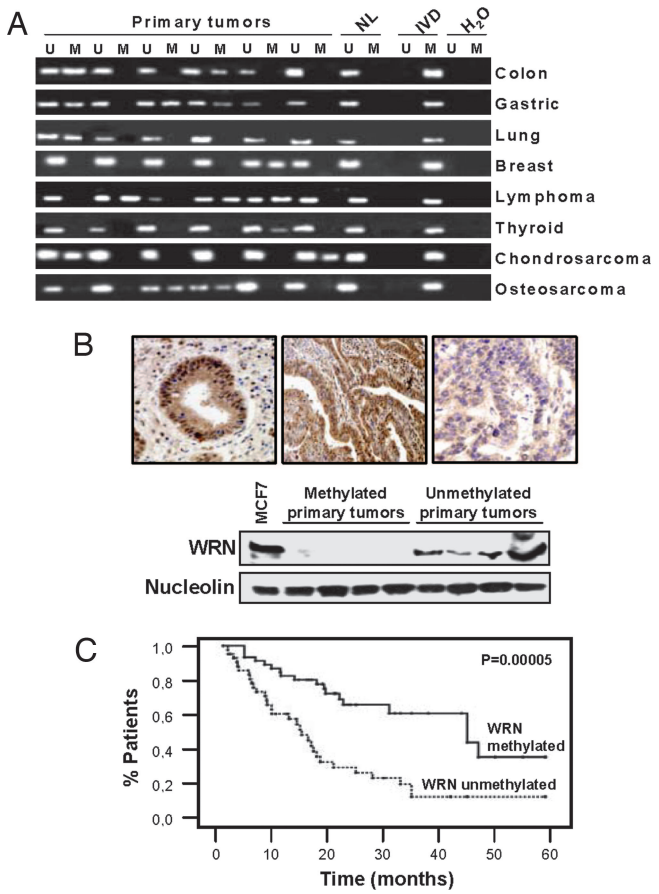

Fig. 4. WRN CpG island hypermethylation in primary human malignancies. (*A*) Analysis of WRN methylation by methylation-specific PCR. The presence of a PCR band under lane M indicates methylated genes. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) are used as negative and positive control for unmethylated and methylated genes, respectively. (*B*) WRN protein expression in primary human tumors. (*Upper*) Immunohistochemistry of WRN in a normal gastric gland (*Left*), unmethylated at WRN, and in an unmethylated gastric tumor showing strong WRN expression (*Center*) and a methylated gastric tumor demonstrating loss of WRN staining (*Right*). (*Lower*) Western blot analyses of colorectal tumors showing the tight association between WRN methylation and loss of expression. (*C*) Kaplan–Meier analysis of WRN promoter hypermethylation in patients with colorectal cancer treated with irinotecan and its impact on survival. A significant increased overall survival is observed in patients with WRN methylation.

rosis starting as early as the second or third decade of life (1, 2). Most important, WS patients have a high incidence of malignant neoplasms (1, 2). What makes the case even more interesting it is that the tumor type of neoplasms appearing in WS patients is remarkably different from that observed in people who do not have the syndrome: the ratio of mesenchymal:epithelial cancers is 1:1, as compared with 1:10 in the normal aging population (12, 32). Thus, it seems that the accelerated aging process in WS patients contributes to the higher incidence of tumors, but the specific loss of the WRN gene confers a particular tumor-type prone phenotype, in a similar fashion to what has been observed with other familial tumor-suppressor genes with DNA-repair function, such as hMLH1 or BRCA1 (33). Our observation that the WRN gene undergoes epigenetic inactivation by CpG island promoter hypermethylation in various tumor types of both mesenchymal and epithelial origin, including those commonly observed in WRN patients (such as osteosarcoma, thyroid, and gastric tumors) (34), may provide further insight into the WRN protein's contribution to the tumorigenic process.

Several new avenues of research now present themselves, such as the links between WRN epigenetic silencing and two critical components of the cellular machinery, telomeres and p53. In the first case, it is known that the WRN protein interacts with several telomere proteins and unwinds a telomeric D-loop structure (35). Furthermore, the loss of WRN function facilitates the activation of the alternative lengthening of telomeres mechanism, which may engender cancer-relevant chromosomal aberrations and tumor formation in mouse models (36). Interestingly, alternative lengthening of telomeres is commonly observed in sarcomas, where we have also observed WRN hypermethylation and which are characteristic of patients with WS. Similar questions can be applied to p53. The induction of apoptosis by p53 is attenuated in WRN-deficient cells (37), and this phenotype cannot be rescued by other RecQ DNA helicases, such as BLM (38). Related to this last issue, it is important to remember that WRN is the only RecQ DNA helicase that, in addition to its helicase activity, has an exonuclease activity (9). Thus, our exonuclease enzymatic determination in WRN-immunoprecipitated cells provides a specific functional assay for the WRN protein against all of the other RecQ DNA helicase family members.

Finally, it is worth emphasizing the potential clinical relevance of our findings. Our observations demonstrate that WRN hypermethylation renders these cancer cells very sensitive to the action of inhibitors of topoisomerase and DNA-damaging agents. This is not an observation merely restricted to laboratory models but one that can be translated to real patients. We have shown that the use of a chemotherapeutic agent with topoisomerase-inhibition activity, irinotecan, is associated with increased overall survival in colorectal patients who display WRN promoter CpG island hypermethylation. Larger prospective studies would be necessary to build on these findings, but it is a line of translational research that merits further exploration.

In summary, our results suggest that the abolition of the tumor-suppressor functions of the progeroid WRN gene by epigenetic silencing is a relevant event in human tumorigenesis, associated with the generation of chromosomal instability. At the same time, it constitutes an Achilles' heel for these tumors, because they become more sensitive to the chemotherapeutic action of topoisomerase inhibitors and DNA-damaging agents. All of these findings together represent another chapter in the story of the close and long established connection between the processes of aging and cancer.

Methods

Cell Lines and Tumor Samples. The 11 human cancer cell lines examined in this study were obtained from the American Type Culture Collection. The cell lines represented three different types of malignancies, colon (HCT-116 and COLO-205), breast (MCF-7 and MDA-MB-231), and leukemia (HL-60, U937, and REH). Cell lines were maintained in appropriate media and treated with 1 μM 5-aza-2′-deoxycytidine (Sigma) for 3 days to achieve demethylation (19, 21, 22). WS $-/-$ cells (AG11395) were obtained from the Coriell Cell Repositories (Camden, NJ). The collection of primary tumor samples analyzed is described in ref. 22.

DNA Methylation Analysis of the WRN Gene. We established WRN CpG island methylation status by PCR analysis of bisulfite-modified genomic DNA. First, methylation status was analyzed by bisulfite genomic sequencing of both strands of the CpG island. The primers used were 5′-AGG TTT TTA GTY GGY GGG TAT TTA-3′ (sense) and 5′-AAC CCC CTC TTC CCC TCA-3′ (antisense), located at -209 bp and $+164$ bp from the transcription start site. The second analysis used methylation-specific PCR with primers specific for either the methylated or modified unmethylated DNA. Primer sequences for the unmethylated reaction were 5′-GTA GTT GGG TAG GGG TAT TGT TTG T-3′ (sense) and 5′-AAA CAA AAT CCA CCA CCC ACC CC-3′ (antisense), and for the meth-

ylated reaction were 5′-CGG GTA GGG GTA TCG TTC GC-3′ (sense) and 5′-AAC GAA ATC CAC CGC CCG CC-3′ (antisense). Primers were located at −36 (sense) and +129 (antisense) from the transcription start site.

WRN RNA and Protein Analysis by Western Blotting, Immunofluorescence, and Immunohistochemistry. RNA was isolated by using TRIzol (Life Technologies). Two micrograms of RNA were reverse-transcribed by using SuperScript II reverse transcriptase (Gibco/BRL) and amplified by using specific primers for WRN (forward, 5′-GCA TGT GTT CGG AAG AGT GTT T-3′; reverse, 5′-TGA CAT GGA AGA AAC GTG GAA-3′). PCR was performed for 25 cycles (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s). Cell lysates for protein analysis were prepared and analyzed by Western blotting with the WRN antibody ab200 (rabbit polyclonal; Abcam). Immunofluorescence and immunohistochemistry experiments were developed as described in refs. 22 and 30.

Exonuclease Assay. Exonuclease enzymatic activity was measured as described in ref. 39, using lysates from 80,000 cells immunoprecipitated with WRN antibody H-300 (rabbit IgG; Santa Cruz Biotechnology). The DNA exonuclease substrate consisted of a double-stranded DNA molecule with one blunt end and one recessed 3′ end (5′ overhang of 20 nucleotides). Reactions were incubated at 37°C for 60 min. The digestion products of these reactions were separated on 15% denaturing polyacrylamide gels and visualized by using a PhosphorImager (Molecular Dynamics).

Flow Cytometry and Cytogenetic Assays. The percentage of apoptotic cells was determined by flow cytometry using the Vybrant apoptosis assay kit #4 (YO-PRO-1/propidium iodide; Molecular Probes/Invitrogen). Briefly, cells were washed twice with ice-cold PBS and resuspended in PBS containing YO-PRO-1/propidium iodide. Apoptotic cells were identified by flow cytometry after incubation for 20 min. For the cytogenetic assay, 100 metaphases were analyzed for each experimental condition.

WRN Transfection and Small Interfering RNA (siRNA) Assay. The WRN coding sequence corresponding to the cDNA from a B-LCL lymphoblastoid-EBV immortalized cell line was amplified by PCR and directly cloned into the pGEM-T Easy Vector (Promega). The WRN insert was subcloned into the pEGFP-C1 expression vector (Invitrogen) and confirmed by sequencing. For transfection experiments, we used the pEGFP-C1 vector containing the WRN gene or the pEGFP-C1 empty vector. Transfection of MDA-MB-231 and U937 cells was performed by electroporating 10^7 cells. Electroporated cells were washed with PBS and seeded with 10^6 cells per ml in fresh medium containing 20% FBS. Transfected cells were selected by the addition of G418 (600 μg/ml). The WRN interference assays were developed by using a siRNA duplex against the WRN gene that recognizes the sequence 5-CAG GTG AAC TTA GGA AAC TTA-3′ (Qiagen). We used Scramble siRNA (Qiagen) as a negative control.

Colony-Formation Assay. Colony formation on methylcellulose medium (StemCell Technologies) was assayed. Transfected cells were added to a medium containing 80% methylcellulose and 20% conditioned medium from MDA-MB-231 cultures and 600 μg/ml G418. The mixture was then placed in a six-well plate and incubated for 15 days. Colonies containing more than 20 cells were scored as positive.

Mouse Xenograft Model. Six-week-old female athymic nude mice nu/nu (Harlan Sprague Dawley, Indianapolis) were used for MDA-MB-231 tumor xenografts. Ten specimen were used. Both flanks of each animal were injected s.c. with 10^7 cells in a total volume of 200 μl of PBS. The right flank was always used for WRN-MDA-MB-231-transfected cells and the left for empty-vector MDA-MB-231 control cells. Tumor development at the site of injection was measured daily.

Statistical Analysis. Contingency tables were analyzed by using Fisher's exact test. Overall survival curves were estimated by using the Kaplan–Meier method and were compared with the log-rank test. All statistical analyses were performed by using SPSS (Version 10.1; SPSS, Chicago).

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