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WRN promoter methylation possibly connects mucinous differentiation, microsatellite instability and CpG island methylator phenotype in colorectal cancer

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Werner syndrome is a premature aging syndrome characterized by early onset of cancer and abnormal cellular metabolism of glycosaminoglycan. The WRN helicase plays an important role in the maintenance of telomere function. *WRN* promoter methylation and gene silencing are common in colorectal cancer with the CpG island methylator phenotype (CIMP), which is associated with microsatellite instability (MSI) and mucinous tumors. However, no study has examined the relationship between mucinous differentiation, *WRN* methylation, CIMP and MSI in colorectal cancer. Utilizing 903 population-based colorectal cancers and real-time PCR (MethyLight), we quantified DNA methylation in *WRN* and eight other promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*) known to be specific for CIMP. Supporting *WRN* as a good CIMP marker, *WRN* methylation was correlated well with CIMP-high diagnosis ($\geq$6/8 methylated promoters), demonstrating 89% sensitivity and 81% specificity. *WRN* methylation was associated with the presence of any mucinous component and $\geq$50% mucinous component ($P<0.0001$). Because both MSI and CIMP were associated with mucinous tumors and *WRN* methylation, we stratified tumors into 9 MSI/CIMP subtypes, to examine whether the relationship between *WRN* methylation and mucin still persisted. In each MSI/CIMP subtype, tumors with mucinous component were persistently more common in *WRN*-methylated tumors than *WRN*-unmethylated tumors ($P=0.004$). No relations of *WRN* methylation with other variables (age, sex, tumor location, poor differentiation, signet ring cells, lymphocytic reactions, *KRAS*, *BRAF*, p53, p21 or 18q loss of heterozygosity) persisted after tumors were stratified by CIMP status. In conclusion, *WRN* methylation is associated with mucinous differentiation independent of CIMP and MSI status. Our data suggest a possible role of *WRN* methylation in mucinous differentiation, and may provide explanation to the enigmatic association between mucin and MSI/CIMP.
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Werner syndrome is a premature aging syndrome characterized by accelerated aging and early onset of cancer and other diseases. *WRN* (the Werner syndrome gene) and its product (DNA helicase[1]) have been shown to be important in maintenance of telomere structures,[2] and initiation of DNA damage response after telomere disruption.[3] Normal telomere function is an important cellular mechanism against aging and manifestations of Werner syndrome.[4–6] WRN can interact with p53,[7,8] and replication protein A1,[9] the latter of which is required for stabilization of single-stranded DNA during DNA replication.[10] Considering the importance of the WRN helicase in the maintenance of telomere function, WRN likely acts as a 'caretaker' tumor suppressor gene for genome integrity. Promoter methylation and gene silence of *WRN* have been shown in cell lines from colon cancer, breast cancer and leukemia.[11] Restoration of WRN

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expression causes reduced colony formation and inhibition of tumor growth in a xenograft model, confirming tumor suppressor property of WRN.[11] *WRN* promoter methylation may predict good survival among colorectal cancer patients treated by irinotecan,[11] which is a topoisomerase inhibitor.

Transcriptional inactivation by cytosine methylation at promoter CpG islands of tumor suppressor genes is an important mechanism in human carcinogenesis.[12–15] A number of tumor suppressor genes are silenced by promoter methylation in colorectal cancers.[12,13] A subset of colorectal cancers exhibit widespread promoter CpG island methylation, which is referred to as the CpG island methylator phenotype (CIMP).[16] CIMP-high colorectal tumors have a distinct clinical, pathologic, and molecular profile, such as associations with proximal tumor location, female sex, poor differentiation, microsatellite instability (MSI), and high *BRAF* and low *TP53* mutation rates.[17–20] In addition, mucinous colorectal carcinomas frequently show the CIMP and MSI phenotypes.[20–23] However, the mechanism of mucinous differentiation is poorly understood.

Werner syndrome patients have been known to demonstrate elevated levels of hyaluronic acid in serum and urine.[24–27] WRN-deficient cells exhibit abnormal metabolism of glycosaminoglycan,[28–31] and in particular, excretion of glycosaminoglycan is increased from WRN-deficient cells.[32] Thus, we hypothesized that *WRN* promoter methylation and gene silencing might, at least in part, explain excessive mucin secretion in a subset of colorectal cancers with CIMP and/or MSI.

In this study, using quantitative DNA methylation analysis (MethyLight technology) and a large number of population-based colorectal cancers, we examined the relationship between *WRN* promoter methylation, CIMP, MSI and mucinous features. We have shown that *WRN* methylation is correlated with mucinous differentiation independent of CIMP and MSI, thus providing a possible explanation to the enigmatic association between mucin and CIMP/MSI.

Materials and methods

Study Group

We utilized the databases of two large prospective cohort studies; the Nurses' Health Study ($N=121\,700$ women followed since 1976),[33] and the Health Professional Follow-up Study ($N=51\,500$ men followed since 1986).[34] Informed consent was obtained from all participants prior to inclusion in the cohorts. A subset of the cohort participants developed colorectal cancers during prospective follow-up. Thus, these colorectal cancers represented population-based, relatively unbiased samples (compared to retrospective or single-hospital-based samples). Previous studies on Nurses' Health Study and Health Professionals Follow-up Study have described baseline characteristics of cohort participants and incident colorectal cancer cases, and confirmed that our colorectal cancer cases were well representative as a population-based sample.[33,34] Clinical features of each colorectal cancer case were obtained by chart review. We collected paraffin-embedded tissue blocks from hospitals where cohort participants with colorectal cancers had undergone resections of primary tumors. We excluded cases if adequate paraffin-embedded tumor tissue was not available at the time of the study. As a result, a total of 903 colorectal cancer cases (405 from men's cohort and 498 from women's cohort) were included. Among our cohort studies, there was no significant difference in demographic features between cases with tissue available and those without available tissue.[35] Many of the cases have been previously characterized for status of CIMP, MSI, *KRAS* and *BRAF*.[23] However, no tumor has been examined for *WRN* methylation in our previous studies. Tissue collection and analyses were approved by the Dana–Farber Cancer Institute and Brigham and Women's Hospital Institutional Review Boards.

Histopathologic Evaluations

Hematoxylin and eosin (H&E) stained tissue sections were examined under a light microscope by one of the investigators (SO) blinded from clinical and other laboratory data. Various pathologic features were examined as described previously.[36] Tumors were classified into well/moderately-differentiated ($<50\%$ solid areas); and poorly-differentiated tumors ($\geq 50\%$ solid areas). The extent and type (intracellular or extracellular) of mucinous component in each tumor were evaluated. In addition, tumor infiltrating lymphocytes, Crohn's-like reaction, and peritumoral lymphocytic reaction have been evaluated, and graded as absent/mild or moderate/severe.[36]

Genomic DNA Extraction and Whole Genome Amplification

Genomic DNA was extracted from dissected tumor tissue sections using QIAmp DNA Mini Kit (Qiagen, Valencia, CA USA) as described previously.[37] Normal DNA was obtained from colonic tissue at resection margins. Whole genome amplification (WGA) of genomic DNA was performed by PCR using random 15-mer primers for subsequent MSI analysis and *KRAS* and *BRAF* sequencing.[37] Previous studies by us and others showed that WGA did not significantly affect *KRAS* mutation detection or microsatellite analysis.[37,38]

Analyses for Microsatellite Instability and 18q Loss of Heterozygosity

Methods to analyze for MSI status have been described previously.[37] In addition to the recommended

MSI panel consisting of D2S123, D5S346, D17S250, BAT25 and BAT26,[39] we also used BAT40, D18S55, D18S56, D18S67 and D18S487 (ie, 10-marker panel).[37] A 'high degree of MSI' (MSI-H) was defined as the presence of instability in $\geq$30% of the markers. A low degree of MSI (MSI-L) was defined as the presence of instability in $<$30% of the markers, and 'microsatellite stable' (MSS) tumors were defined as tumors without an unstable marker.

18q loss of heterozygosity (LOH) analysis using microsatellite markers D18S55, D18S56, D18S67 and D18S487 were performed as described previously.[37] We duplicated PCR and electrophoresis in each sample to exclude allele dropouts of one of two alleles. Loss of heterozygosity at each locus was defined as 40% or greater reduction of one of two allele peaks in tumor DNA relative to normal DNA.

Sequencing of *KRAS* and *BRAF*

Methods of PCR and sequencing targeted for *KRAS* codons 12 and 13, and *BRAF* codon 600 have been described previously.[37] Pyrosequencing was performed using the PSQ96 HS System (Biotage AB and Biosystems, Uppsala, Sweden) according to the manufacturer's instructions.

Real-Time PCR (MethyLight) for Quantitative DNA Methylation Analysis

Sodium bisulfite treatment on genomic DNA was performed as described previously.[40] Real-time PCR to measure DNA methylation (MethyLight) was performed as described previously.[41] Utilizing ABI 7300 (Applied Biosystems, Foster City, CA, USA), we examined *WRN* promoter and eight other CIMP-specific promoters (*CACNA1G*, *CDKN2A (p16)*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*).[19,23] We have shown that these eight markers are sensitive and specific markers for CIMP diagnosis.[23] *COL2A1* (the collagen 2A1 gene) was used to normalize for the amount of input bisulfite-converted DNA.[40] PCR primers and probe for *WRN* were (bisulfite-converted nucleotides are highlighted by bold face and italics): WRN-F, 5′-G***T***A TCG ***TT***C GCG GCG ***TTT*** A***T***-3′ (Genbank No AY442327, nucleotide Nos 1827–1846); WRN-R, 5′-***A***CG ***AAA*** CCG ***A***T***A*** TCC GAA ***A***TC A -3′ (nucleotide Nos 1887–1908); WRN-probe, 6FAM-5′-***TTT T***T***T*** TG CGG ***T***CG ***T***TG CGG G-3′-BHQ-1 (nucleotides 1855–1876). The *WRN* promoter CpG island that we examined is the one which was analyzed by Agrelo *et al*.[11] All other primers and probes were described previously.[19] The percentage of methylated reference (PMR, ie, degree of methylation) at a specific locus was calculated by dividing the *GENE:COL2A1* ratio of template amounts in a sample by the *GENE:COL2A1* ratio of template amounts in *Sss*I-treated human genomic DNA (presumably fully methylated) and multiplying this value by 100.[41] A PMR cutoff value of 4 (except for 6 in *CRABP1* and *IGF2*, and 10 for WRN) was based on previously validated data.[40] Precision and performance characteristics of bisulfite conversion and subsequent MethyLight assays have been previously evaluated and the assays have been validated.[40] CIMP-high was defined as the presence of $\geq$6/8 methylated promoters, CIMP-low as 1/8–5/8 methylated promoters and CIMP-0 as the absence (0/8) of methylated promoters, according to the previously established criteria.[23]

Tissue Microarrays and Immunohistochemistry for p53 and p21 (CDKN1A)

Tissue microarrays were constructed as described previously,[35] using the Automated Arrayer (Beecher Instruments, Sun Prairie, WI, USA). We examined two to four tumor tissue cores for each marker. A previous validation study has shown that examining two TMA cores can yield comparable results to examining whole tissue sections in more than 95% of cases.[42] We examined whole tissue sections for cases in which no tissue block was available for TMA construction or results were equivocal in TMAs. Immunohistochemistry for p53 was performed as described previously.[37] p53 positivity was defined as 50% or more of tumor cells with unequivocal strong nuclear staining, as this high threshold considerably improved specificity in previous studies.[43,44]

For p21 (CDKN1A/CIP1) immunohistochemistry, we incubated deparaffinized whole tissue sections in citrate buffer at high power in a microwave for 30 min (in a pressure cooker). Tissue sections were then incubated with 3% H_2O_2 (10 min) to block endogenous peroxidase, and then incubated with protein block (Vector Laboratories, Burlingame, CA, USA) (10 min). Primary anti-p21 antibody (Pharmingen, San Diego, CA, USA) (dilution 1:50) was applied for 30 min at room temperature. Then, biotinylated secondary multilink antibody (Biogenex, San Ramon, CA, USA) was applied (20 min), horse radish peroxidase avidin complex (Biogenex) was added and sections were visualized by DAB (30 s) and methyl-green counterstain. p21 loss was defined as less than 5% of tumor cells with nuclear staining.

Appropriate positive and negative controls were included in each run of immunohistochemistry. All immunohistochemically-stained slides were interpreted by one of the investigators (SO) blinded from any other clinical and laboratory data.

Statistical Analysis

In statistical analysis, χ^2 test (or Fisher's exact test when the number in any category was less than 10) was performed for categorical data, and kappa coefficients were calculated to determine the degree

of agreement between two observers, using SAS program (Version 9.1, SAS Institute, Cary, NC, USA). All *P*-values were two-sided, and statistical significance was set at $P \leq 0.05$.

Results

WRN Promoter Methylation and CIMP in Colorectal Cancer

Utilizing MethyLight technology, we quantified DNA methylation in *WRN* and a panel of eight promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*). The latter eight promoters constitutes a sensitive and specific marker panel for CIMP-high.[23] Among the 903 tumors, 266 (29%) were positive for *WRN* promoter methylation. *WRN* methylation was slightly more common in women (32% = 159/498) than in men (26% = 107/405) though the difference was not statistically significant. Sensitivity and specificity of *WRN* methylation for the diagnosis of CIMP-high (≥6/8 methylated promoters, not including *WRN*) were 89 and 81%, respectively (Table 1), indicating that *WRN* was a good marker for CIMP-high (slightly better than *CDKN2A*[23]). This fact also indicates that CIMP status is a confounding factor when one analyzes the relationship between *WRN* methylation and any clinicopathologic or molecular variables. Thus, in subsequent analyses, we stratified tumors according to *WRN* and CIMP status (as in Table 2 and Table 3). Because 5/8 methylated tumors show borderline features between CIMP-high and CIMP-low,[23] those were excluded from further analyses.

We also quantified *WRN* methylation in normal colon tissue from eight *WRN*-methylated tumor cases and seven *WRN*-unmethylated tumor cases. Only one normal sample from the eight *WRN*-methylated tumor cases showed *WRN* methylation, and no normal sample from the seven *WRN*-unmethylated tumor cases showed *WRN* methylation.

WRN Methylation is Associated with Mucinous Differentiation Independent of CIMP Status

Table 2 summarizes the relations between *WRN* methylation and clinical and pathologic features in colorectal cancer. The presence of any mucinous component was significantly correlated with *WRN* methylation in all cases ($P < 0.0001$), in CIMP-high tumors ($P = 0.04$), and in CIMP-low/0 tumors ($P < 0.0001$). The frequencies of both 1–49% mucinous tumors and ≥50% mucinous tumors were higher in *WRN*-methylated tumors than *WRN*-unmethylated tumors, regardless of CIMP status (Table 2).

Correlations of *WRN* Methylation with Other Clinicopathologic and Molecular Features

Proximal tumor location, poor differentiation, signet ring cells, tumor infiltrating lymphocytes, Crohn's-like reaction and peritumoral lymphocytic reaction were associated with *WRN* methylation in all cases, but no significant correlations persisted after tumors were stratified by CIMP status (Table 2), indicating these features were associated primarily with CIMP, but not directly with *WRN* methylation. There was no significant correlation between *WRN* and age at diagnosis.

Table 3 summarizes the relations between *WRN* methylation and molecular alterations in colorectal cancer. *WRN* methylation was correlated with MSI-H, *BRAF* mutation, 18q loss of heterozygosity negativity, p53 negativity and intact p21 expression in all cases, but no significant relationship persisted after tumors were stratified by CIMP status.

Relationship Between *WRN* Methylation and Mucinous Differentiation Persisted in Each MSI/CIMP Subtype

Because mucinous features are correlated with both MSI-H and CIMP-high, we stratified tumors into nine MSI/CIMP subtypes and examined the frequency of mucinous differentiation according to *WRN* methylation status (Figure 1). There was no MSI-L CIMP-high *WRN*-unmethylated tumor. After exclusion of MSI-L/CIMP-high, within each of the remaining eight MSI/CIMP subtypes, *WRN*-methylated tumors consistently exhibited higher frequencies of both ≥50% mucinous tumors and tumors with any mucinous component. Under the null hypothesis that *WRN* methylation was unrelated

Table 1 Sensitivity and specificity of *WRN* methylation for the diagnosis of CIMP-high

	CIMP-high (≥6/8 methylated promoters)	*Non-CIMP-high (≤5/8 methylated promoters)*	*Total*
WRN methylation			
Positive	119 (sensitivity 89%[a])	141 (19%)	260
Negative	14 (11%)	602 (specificity 81%[b])	616
Total	133	743	876

Abbreviation: CIMP, CpG island methylator phenotype.
[a]Sensitivity is defined as the number of *WRN*-positive CIMP-high cases divided by the number of all CIMP-high cases.
[b]Specificity is defined as the number of *WRN*-negative non-CIMP-high cases divided by the number of all non-CIMP-high cases.

Table 2 Frequencies of specific clinical and pathologic features in colorectal cancer according to *WRN* methylation and CIMP status

Clinical and pathologic features	*Total* N	*All cases* *WRN methylation*		P-*value*	*CIMP-high* *WRN methylation*		P-*value*	*CIMP-low/0* *WRN methylation*		P-*value*
		(+)	*(−)*		*(+)*	*(−)*		*(+)*	*(−)*	
All cases	903	266	637		119	14		128	615	
Men	405	107	298		41	2		56	293	
Women	498	159	339		78	12		72	322	
Age										
Total examined	866	258	608		116	14		123	586	
<60	196	43 (17%)	153 (25%)		9 (7.8%)	1 (7.1%)		28 (23%)	151 (26%)	
60–69	370	120 (47%)	250 (41%)		57 (49%)	6 (43%)		57 (46%)	239 (41%)	
≥70	300	95 (37%)	205 (34%)		50 (43%)	7 (50%)		38 (31%)	196 (33%)	
Tumor location										
Total examined	522	167	355		71	10		82	339	
Proximal	248	125 (75%)	123 (35%)	<0.0001	65 (92%)	10 (100%)		48 (59%)	111 (33%)	<0.0001
Distal	274	42 (25%)	232 (65%)		6 (8.5%)	0		34 (41%)	228 (67%)	
Tumor differentiation										
Total examined	885	264	621		119	14		126	600	
Well/moderate	802	221 (84%)	581 (94%)	<0.0001	83 (70%)	10 (71%)		120 (95%)	566 (94%)	
Poor	83	43 (16%)	40 (6.4%)		36 (30%)	4 (29%)		6 (4.8%)	34 (5.7%)	
Mucinous/signet ring cell features										
Total examined	782	247	535		111	14		118	515	
Non-mucinous carcinoma	471	98 (40%)	373 (70%)	**<0.0001**	37 (33%)	9 (64%)	**0.04**	54 (46%)	361 (70%)	**<0.0001**
Mucinous 1–100%	311	149 (60%)	162 (30%)		74 (67%)	5 (36%)		64 (54%)	154 (30%)	
1–49%	191	81 (33%)	110 (21%)		34 (31%)	2 (14%)		39 (33%)	106 (21%)	
≥50%	120	68 (28%)	52 (9.7%)		40 (36%)	3 (21%)		25 (21%)	48 (9.3%)	
Non-signet ring call carcinoma	721	214 (87%)	507 (95%)	<0.0001	90 (81%)	11 (79%)		108 (92%)	490 (95%)	
Signet ring cells 1–100%	61	33 (13%)	28 (5.2%)		21 (19%)	3 (21%)		10 (8.5%)	25 (4.9%)	
1–49%	46	29 (12%)	17 (3.2%)		18 (16%)	3 (21%)		9 (7.6%)	14 (2.7%)	
≥50%	15	4 (1.6%)	11 (2.1%)		3 (2.7%)	0		1 (0.8%)	11 (2.1%)	
Tumor infiltrating lymphocytes										
Total examined	877	258	619		116	14		124	597	
Absent/mild	778	195 (76%)	583 (94%)	<0.0001	69 (59%)	10 (71%)		111 (90%)	567 (95%)	0.02
Moderate/severe	99	63 (24%)	36 (5.8%)		47 (41%)	4 (29%)		13 (10%)	30 (5.0%)	
Crohn's-like reaction										
Total examined	674	197	477		87	12		94	458	
Absent/mild	611	158 (80%)	453 (95%)	<0.0001	56 (64%)	10 (83%)		88 (94%)	436 (95%)	
Moderate/severe	63	39 (20%)	24 (5.0%)		31 (36%)	2 (17%)		6 (6.4%)	22 (4.8%)	
Peritumoral lymphocytic reaction										
Total examined	879	259	620		116	14		125	598	
Absent/mild	782	211 (81%)	571 (92%)	<0.0001	83 (72%)	11 (79%)		112 (90%)	553 (92%)	
Moderate/severe	97	48 (19%)	49 (7.9%)		33 (28%)	3 (21%)		13 (10%)	45 (7.5%)	

Abbreviation: CIMP, CpG island methylator phenotype.
Only significant *P*-values are described.
Bold values indicate significant *P*-values across any CIMP types.

Table 3 Frequencies of specific molecular features in colorectal cancer according to *WRN* methylation and CIMP status

Molecular features	*Total* N	*All cases* *WRN methylation*		P-*value*	*CIMP-high* *WRN methylation*		P-*value*	*CIMP-low/0* *WRN methylation*		P-*value*
		(+)	*(−)*		*(+)*	*(−)*		*(+)*	*(−)*	
MSI										
Total examined	874	261	613		117	14		125	591	
MSI-H	127	89 (34%)	38 (6.2%)	<0.0001	80 (68%)	10 (71%)		9 (7.2%)	27 (4.6%)	
MSI-L/MSS	747	172 (66%)	575 (94%)		37 (32%)	4 (29%)		116 (93%)	564 (95%)	
KRAS										
Total examined	860	254	606		114	14		121	584	
Mutant	313	96 (38%)	217 (36%)		16 (14%)	2 (14%)		71 (59%)	212 (36%)	<0.0001
Wild-type	547	158 (62%)	389 (64%)		98 (86%)	12 (86%)		50 (41%)	372 (64%)	
BRAF										
Total examined	860	254	606		114	14		121	584	
Mutant	112	82 (32%)	30 (5.0%)	<0.0001	68 (60%)	9 (64%)		7 (5.8%)	19 (3.3%)	
Wild-type	748	172 (68%)	576 (95%)		46 (40%)	5 (36%)		114 (94%)	565 (97%)	
18q LOH (only non-MSI-H cases)										
Total examined	379	87	292		18	3		59	283	
(+)	235	44 (51%)	191 (65%)	0.01	8 (44%)	1 (33%)		33 (56%)	188 ((66%)	
(−)	144	43 (49%)	101 (35%)		10 (56%)	2 (67%)		26 (44%)	95 (34%)	
p53[a]										
Total examined	891	260	631		119	14		123	609	
(+)	386	82 (32%)	304 (48%)	<0.0001	27 (23%)	3 (21%)		46 (37%)	298 (49%)	0.02
(−)	505	178 (68%)	327 (52%)		92 (77%)	11 (79%)		77 (63%)	311 (51%)	
p21[a]										
Total examined	864	252	612		115	14		120	590	
Loss	508	101 (40%)	407 (67%)	<0.0001	28 (24%)	3 (21%)		63 (52%)	400 (68%)	0.001
(+)	356	151 (60%)	205 (33%)		87 (76%)	11 (79%)		57 (48%)	190 (32%)	

Abbreviations: CIMP, CpG island methylator phenotype; LOH, loss of heterozygosity; MSI, microsatellite instability.
Only significant *P*-values are described.

[a]p53 and p21 status was determined by immunohistochemistry.

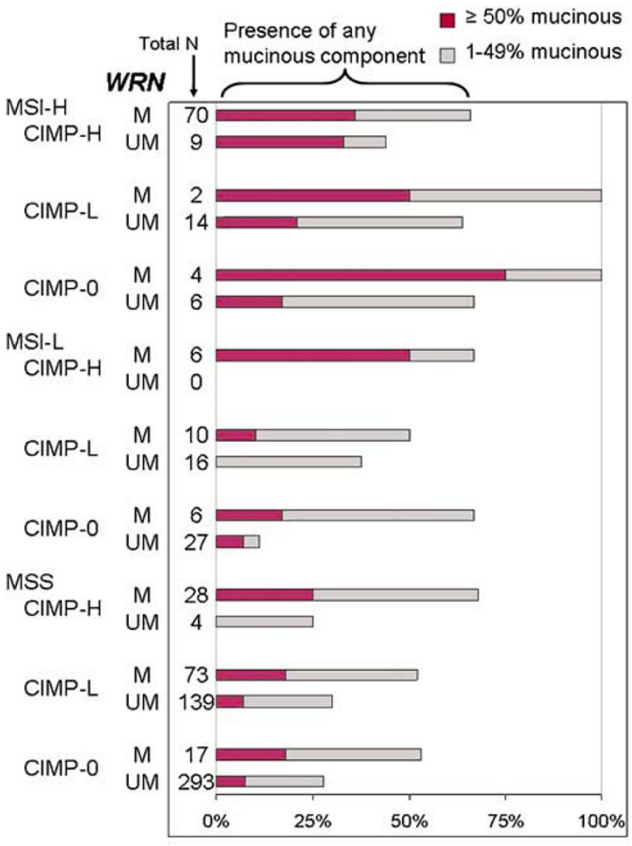

Figure 1 Frequencies of mucinous tumors in *WRN*-methylated and *WRN*-unmethylated colorectal cancers within each of the 9 MSI/CIMP subtypes. Note that *WRN*-methylated tumors show consistently higher frequencies of mucinous differentiation (both ≥50% mucinous tumors and any mucinous tumors) than *WRN*-unmethylated tumors ($P=0.004$). Abbreviations: CIMP, CpG island methylator phenotype; M, methylated; MSI, microsatellite instability; MSS, microsatellite stable; UM, unmethylated.

with mucinous features, the probability that *WRN*-methylated tumors showed a higher frequency of mucinous tumors within one MSI/CIMP group by chance would be 1/2; thus, the statistical significance level for our consistent observations in all of the eight MSI/CIMP categories was $P=(1/2)^8=0.004$. These results implied that *WRN* methylation was associated with mucinous differentiation independent of MSI and CIMP.

Discussion

We conducted this study to examine the relationship between mucinous differentiation and promoter methylation in *WRN* (the Werner syndrome gene). We have found that, compared to *WRN*-unmethylated colorectal cancers, *WRN*-methylated tumors consistently show higher frequencies of both ≥50% mucinous tumors and tumors with any mucinous component, within each MSI/CIMP subtype. Thus, *WRN* methylation appears to be correlated with mucinous differentiation regardless of CIMP and MSI status. Considering the relation between *WRN* methylation and CIMP/MSI, and the link between Werner syndrome and abnormal glycosaminoglycan metabolism, our data suggest the possibility that the enigmatic association between mucinous differentiation and CIMP/MSI may be connected by *WRN* methylation.

We utilized quantitative DNA methylation assays (MethyLight), which is robust and can reproducibly differentiate low-level methylation from high-level methylation.[40] Our resource of a large number of colorectal cancers, derived from two large prospective cohorts (relatively unbiased samples compared to retrospective or single-hospital-based samples), has enabled us to precisely estimate the frequency of colorectal cancers with a specific molecular feature (eg, *WRN* methylation, MSI-H, etc). The large number of samples has also provided a sufficient power to examine the relation between *WRN* methylation and mucinous features in rare tumor subtypes, such as MSI-H CIMP-0, MSI-L CIMP-low, etc.

The association between *WRN* methylation and mucinous features in colorectal cancer is intriguing, and it appears to be independent of CIMP and MSI status. The presence of mucinous component (even with a minor component) in colorectal cancer implies specific molecular pathologic features, including associations with MSI-H, *BRAF* mutation, *KRAS* mutation, p53 negativity and fatty acid synthase overexpression.[37] However, pathogenetic mechanism of mucinous differentiation is poorly understood. Abnormal glycosaminoglycan metabolism is present in Werner syndrome cells,[28–31] and Werner syndrome patients show elevated levels of hyaluronic acid in serum and urine.[24–27] Thus, it is possible that abnormal glycosaminoglycan metabolism may cause mucin overproduction in colorectal cancer with *WRN* methylation and functional loss. This hypothesis can, at least in part, explain the well-known association between mucinous differentiation and MSI/CIMP in colorectal cancer,[20–23] as we have shown the positive correlations between *WRN* methylation and MSI/CIMP, and between *WRN* methylation and mucinous differentiation.

Our data also indicate that *WRN* methylation can serve as a good marker for the diagnosis of CIMP-high with 89% sensitivity and 81% specificity. We have previously shown that all of the eight promoters including *RUNX3*, *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, *SOCS1* and *CDKN2A* exhibit good sensitivity and specificity, and thus can be used as a CIMP-high diagnostic panel.[23] In fact, *WRN* shows slightly superior performance to *CDKN2A* (with sensitivity 85% and specificity 81% when *CDKN2A* is excluded from the CIMP panel[23]). Thus, *WRN* can be included in a methylation maker panel for CIMP-high diagnosis.

In summary, *WRN* promoter methylation in colorectal cancer is associated with mucinous differentiation independent of MSI and CIMP status. *WRN* methylation may connect the enigmatic link

between mucinous differentiation and MSI/CIMP. Further studies are necessary to elucidate the exact pathogenic mechanism of mucinous differentiation in colorectal cancer.

Note added in proof

Detailed methods were previously described as follows: the selection of CDKN2A, CRABP1 and MLH1 as CIMP panel markers,[45] whole genome amplification and KRAS Pyrosequencing,[46] BRAF Pyrosequencing,[47] and p53 immunohistochemistry.[48]

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References

1 Gray MD, Shen JC, Kamath-Loeb AS, *et al.* The Werner syndrome protein is a DNA helicase. Nat Genet 1997;17:100–103.
2 Multani AS, Chang S. WRN at telomeres: implications for aging and cancer. J Cell Sci 2007;120:713–721.
3 Eller MS, Liao X, Liu S, *et al.* A role for WRN in telomere-based DNA damage responses. Proc Natl Acad Sci USA 2006;103:15073–15078.
4 Shay JW, Wright WE. Hallmarks of telomeres in ageing research. J Pathol 2007;211:114–123.
5 Martin JE, Sheaff MT. The pathology of ageing: concepts and mechanisms. J Pathol 2007;211:111–113.
6 Chang S, Multani AS, Cabrera NG, *et al.* Essential role of limiting telomeres in the pathogenesis of Werner syndrome. Nat Genet 2004;36:877–882.
7 Sommers JA, Sharma S, Doherty KM, *et al.* p53 modulates RPA-dependent and RPA-independent WRN helicase activity. Cancer Res 2005;65:1223–1233.
8 Wirtenberger M, Frank B, Hemminki K, *et al.* Interaction of Werner and Bloom syndrome genes with p53 in familial breast cancer. Carcinogenesis 2006;27: 1655–1660.
9 Brosh Jr RM, Orren DK, Nehlin JO, *et al.* Functional and physical interaction between WRN helicase and human replication protein A. J Biol Chem 1999;274: 18341–18350.
10 Bochkarev A, Pfuetzner RA, Edwards AM, *et al.* Structure of the single-stranded DNA-binding domain of replication protein A bound to DNA. Nature 1997;385:176–181.
11 Agrelo R, Cheng WH, Setien F, *et al.* Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci USA 2006;103:8822–8827.
12 Laird PW. Cancer epigenetics. Hum Mol Genet 2005;14Spec No 1: R65–R76.
13 Agrawal A, Murphy RF, Agrawal DK. DNA methylation in breast and colorectal cancers. Mod Pathol 2007;20:711–721.
14 Dong SM, Lee EJ, Jeon ES *et al.* Progressive methylation during the serrated neoplasia pathway of the colorectum. Mod Pathol 2005;18:170–178.
15 Kawaguchi K, Oda Y, Saito T, *et al.* DNA hypermethylation status of multiple genes in soft tissue sarcomas. Mod Pathol 2006;19:106–114.
16 Toyota M, Ahuja N, Ohe-Toyota M, *et al.* CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA 1999;96:8681–8686.
17 Hawkins N, Norrie M, Cheong K, *et al.* CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology 2002;122:1376–1387.
18 Whitehall VL, Wynter CV, Walsh MD, *et al.* Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. Cancer Res 2002;62:6011–6014.
19 Weisenberger DJ, Siegmund KD, Campan M, *et al.* CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 2006;38:787–793.
20 Samowitz W, Albertsen H, Herrick J, *et al.* Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 2005;129:837–845.
21 Tanaka H, Deng G, Matsuzaki K, *et al.* BRAF mutation, CpG island methylator phenotype and microsatellite instability occur more frequently and concordantly in mucinous than non-mucinous colorectal cancer. Int J Cancer 2006;118:2765–2771.
22 Alexander J, Watanabe T, Wu TT, *et al.* Histopathological identification of colon cancer with microsatellite instability. Am J Pathol 2001;158:527–535.
23 Ogino S, kawasaki T, Kirkner GJ, *et al.* Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 2007;9:305–314.
24 Murata K. The molecular weight-dependent distribution of urinary glycosaminoglycans in Werner's syndrome. Biochem Med 1985;34:251–258.
25 Tanabe M, Goto M. Elevation of serum hyaluronan level in Werner's syndrome. Gerontology 2001;47: 77–81.
26 Kieras FJ, Brown WT, Houck Jr GE, *et al.* Elevation of urinary hyaluronic acid in Werner's syndrome and progeria. Biochem Med Metab Biol 1986;36: 276–282.
27 Gawkrodger DJ, Priestley GC, Vijayalaxmi, *et al.* Werner's syndrome. Biochemical and cytogenetic studies. Arch Dermatol 1985;121:636–641.
28 Tajima T, Watanabe T, Iijima K, *et al.* The increase of glycosaminoglycan synthesis and accumulation on the cell surface of cultured skin fibroblasts in Werner's syndrome. Exp Pathol 1981;20:221–229.
29 Murata K, Kudo M, Matsumura T. Acidic glycosaminoglycans of SV40-transformed Werner's syndrome cells. Adv Exp Med Biol 1985;190:607–612.

30 Fujiwara Y, Ichihashi M. Glycosaminoglycan synthesis in untransformed and transformed Werner syndrome fibroblasts: a preliminary report. Adv Exp Med Biol 1985;190:613–625.
31 Higuchi T, Ishikawa O, Hayashi H, *et al.* Disaccharide analysis of the skin glycosaminoglycans in patients with Werner's syndrome. Clin Exp Dermatol 1994;19:487–491.
32 Nakamura T, Takagaki K, Kubo K, *et al.* Hyaluronate synthesized by cultured skin fibroblasts derived from patients with Werner's syndrome. Biochim Biophys Acta 1992;1139:84–90.
33 Colditz GA, Hankinson SE. The Nurses' Health Study: lifestyle and health among women. Nat Rev Cancer 2005;5:388–396.
34 Giovannucci E, Liu Y, Rimm EB, *et al.* Prospective Study of predictors of vitamin D status and cancer incidence and mortality in men. J Natl Cancer Inst 2006;98:451–459.
35 Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. New Engl J Med 2007;356:2131–2142.
36 Ogino S, Odze RD, Kawasaki T, *et al.* Correlation of pathologic features with CpG island methylator phenotype (CIMP) by quantitative DNA methylation analysis in colorectal carcinoma. Am J Surg Pathol 2006;30: 1175–1183.
37 Ogino S, Brahmandam M, Cantor M, *et al.* Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. Mod Pathol 2006;19:59–68.
38 Dietmaier W, Hartmann A, Wallinger S, *et al.* Multiple mutation analyses in single tumor cells with improved whole genome amplification. Am J Pathol 1999;154: 83–95.
39 Boland CR, Thibodeau SN, Hamilton SR, *et al.* A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248–5257.
40 Ogino S, kawasaki T, Brahmandam M, *et al.* Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 2006;8:209–217.
41 Eads CA, Danenberg KD, Kawakami K, *et al.* MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32.
42 Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000;80:1943–1949.
43 Curtin K, Slattery ML, Holubkov R, *et al.* p53 alterations in colon tumors: a comparison of SSCP/ sequencing and immunohistochemistry. Appl Immunohistochem Mol Morphol 2004;12:380–386.
44 Hall PA, McCluggage WG. Assessing p53 in clinical contexts: unlearned lessons and new perspectives. J Pathol 2006;208:1–6.
45 Ogino S, Cantor M, Kawasaki T, *et al.* CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. Gut 2006;55: 1000–1006.
46 Ogino S, Kawasaki T, Brahmandam M, *et al.* Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 2005;7:413–421.
47 Ogino S, Kawasaki T, Kirkner GJ, *et al.* CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 2006;8:582–588.
48 Ogino S, Kawasaki T, Kirkner GJ, *et al.* Loss of nuclear p27 (CDKN1B/KIP1) in colorectal cancer is correlated with microsatellite instability and CIMP. Mod Pathol 2007;20:15–22.