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Altered RECQ Helicase Expression in Sporadic Primary Colorectal Cancers[1,2]

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Abstract

Deregulation of DNA repair enzymes occurs in cancers and may create a susceptibility to chemotherapy. Expression levels of DNA repair enzymes have been shown to predict the responsiveness of cancers to certain chemotherapeutic agents. The RECQ helicases repair damaged DNA including damage caused by topoisomerase I inhibitors, such as irinotecan. Altered expression levels of these enzymes in colorectal cancer (CRC) may influence the response of the cancers to irinotecan. Thus, we assessed RECQ helicase (WRN, BLM, RECQL, RECQL4, and RECQL5) expression in primary CRCs, matched normal colon, and CRC cell lines. We found that *BLM* and *RECQL4* mRNA levels are significantly increased in CRC ($P = .0011$ and $P < .0001$, respectively), whereas *RECQL* and *RECQL5* are significantly decreased ($P = .0103$ and $P = .0029$, respectively). RECQ helicase expression patterns varied between specific molecular subtypes of CRCs. The mRNA and protein expression of the majority of the RECQ helicases was closely correlated, suggesting that altered mRNA expression is the predominant mechanism for deregulated RECQ helicase expression. Immunohistochemistry localized the RECQ helicases to the nucleus. RECQ helicase expression is altered in CRC, suggesting that RECQ helicase expression has potential to identify CRCs that are susceptible to specific chemotherapeutic agents.

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[2]This article refers to supplementary materials, which are designated by Tables W1 to W5 and Figures W1 to W3 and are available online at www.transonc.com.

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Introduction

Colorectal cancer (CRC) is a leading cause of cancer deaths in the United States, affecting more than 140,000 people annually [1]. The majority of people with CRC are diagnosed with advanced stage disease that will require adjuvant chemotherapy in addition to surgical resection of the primary cancer [2]. Currently, pathologic stage is the best prognostic factor available and is used to identify patients who are most likely to benefit from adjuvant chemotherapy and/or radiation therapy [3]. Although pathologic stage is the best predictive indicator for determining response to adjuvant therapy, 30% to 40% of presumably cured patients develop recurrent disease [3]. Therefore, there is a need for markers that more accurately identify patients who would benefit from more aggressive adjuvant therapy and for markers that identify the most effective agents for treating a specific patient's cancer.

Molecular alterations in CRC, such as altered gene expression levels, have the potential to be more accurate than pathologic stage for predicting response to treatment. Molecular alterations are likely more accurate predictive markers than TNM stage, because they often directly affect biologic processes that mediate the manner in which cancer cells respond to specific chemotherapeutic agents. These alterations often affect processes central to the mechanisms of action of chemotherapeutic agents, such as the regulation of DNA repair and genomic stability, thereby affecting the cancer's response to chemotherapy [4,5].

Genomic instability is one of the hallmarks of cancer and results from the loss of the ability to maintain the integrity of the genome. Loss of genomic stability results in increased mutation rates in cancer cells and appears to play a prominent role in the response of cancers to chemotherapy [4,6]. DNA damage, if left unrepaired, contributes to genomic instability. DNA damage occurs on a regular basis in cells as a consequence of exposure to environmental mutagens and cellular metabolites. The integrity of the genome is normally maintained by DNA repair enzymes, which can be grouped into functional categories depending on the type of DNA damage they repair. Their dysfunction can contribute to altered DNA processing, genomic instability, and increased susceptibility to aberrant genetic alterations [7,8]. Importantly, recent *in vitro* studies have shown that cancers with loss of function of DNA repair enzymes have an increased susceptibility to chemotherapeutic agents that induce the type of damage normally repaired by those enzymes [7,9]. These studies as well as the successful proof-of-principle studies involving poly (ADP-ribose) polymerase (PARP) inhibitors in BRCA1-deficient tumors [10,11] have led to an intense investigation of DNA repair enzymes as biomarkers in cancers.

From a clinical vantage, the altered expression of DNA repair enzymes has the potential to be used for predicting the response of a cancer to specific chemotherapeutic agents. The differential response of CRCs to DNA-damaging chemotherapeutic agents may reflect the specific DNA repair capabilities of the cancers. Studies have shown that the currently recognized molecular subtypes of CRC can have different clinical behaviors with regard to treatment outcomes. CRCs demonstrating microsatellite instability (MSI) secondary to DNA mismatch repair deficiency have reduced response to 5-fluorouracil [12,13], whereas this same subtype may be more responsive to irinotecan-containing regimens [14]. It is not yet fully understood what drives this variability in clinical response to chemotherapy; however, it is likely rooted in the molecular heterogeneity of each cancer. The variability in clinical response to DNA-damaging chemotherapeutic agents may be driven by heterogeneity in the expression or function of key DNA repair enzymes.

The RECQ helicases are a family of DNA repair enzymes that unwind double-stranded nucleic acids and have a central role in maintaining genomic integrity [15]. The RECQ helicase family includes five ATP-dependent enzymes (WRN, BLM, RECQL, RECQL4, and RECQL5) that have a conserved 3′ to 5′ helicase domain and two additional conserved regions, the RQC and HRDC regions [15]. They modulate complexes involved in homologous recombination, replication fork migration, translocation, and the structure and function of nucleoprotein filaments. They also process topoisomerase I–mediated DNA damage induced by camptothecin derivatives, such as irinotecan [16]. Autosomal recessive loss of function of three of these DNA-modulating enzymes, WRN, BLM and RECQL4, results in human syndromes characterized by constitutional genomic instability and premature aging [17]. These syndromes have also been linked to increased cancer susceptibility, including CRC, making the function of these helicases a point of interest in the pathogenesis of CRC. Furthermore, deficiencies in RECQ helicases have also been shown to result in a cancer-prone phenotype in mice [18,19].

Because the RECQ helicases play a significant role in the maintenance of genomic stability and DNA repair, altered expression of the RECQ helicases may play a role in the pathogenesis of CRC. Furthermore, RECQ helicase expression may be useful for identifying CRCs that are susceptible to specific DNA-damaging chemotherapeutic agents, including those drugs commonly used for the treatment of CRC, 5-fluorouracil, oxaliplatin, and irinotecan [20–22]. Both WRN- and BLM-depleted cells have impaired survival after treatment with the DNA-damaging agents cisplatinum and 5-fluorouracil as well as camptothecin [23]. RECQL5-deficient cell lines show increased sensitivity to camptothecin [20]. Overexpression of one or more of these helicases may mediate resistance to DNA-damaging chemotherapeutic agents, whereas underexpression may identify cancers that are particularly susceptible to these agents. We hypothesized that the expression of the RECQ helicases would differ in primary CRCs compared to matched normal colonic mucosa. Furthermore, we also hypothesized that the expression of the RECQ helicases may differ among the molecular subtypes of CRC. We used quantitative reverse transcription–polymerase chain reaction (qRT-PCR) as well as Western blot analysis and immunohistochemistry (IHC) to assess our hypothesis and found that BLM and RECQL4 expression are increased in CRC, whereas RECQL and RECQL5 expression are decreased. We also show that there are characteristic RECQ helicase expression patterns for different molecular subtypes of CRC. The RECQ helicases are plausible candidates for the development of biomarkers that may predict the therapeutic response of CRCs to chemotherapeutic agents and may be important in CRC pathogenesis.

Materials and Methods

CRC Cases

A total of 46 sporadic primary CRC cases with matched normal colon was assayed. Twenty-six primary CRC cases with matched normal colon tissue were obtained from the Cooperative Human Tissue Network (CHTN) as fresh frozen tissue. An additional 20 primary CRC cases with matched normal colon tissue were obtained in the form of first-strand cDNA (TissueScan Colon Cancer Tissue qPCR Panel III) from OriGene (Rockville, MD). All cases were composed of >60% tumor epithelium.

Human CRC Tissue Microarrays

CRC tissue microarrays (TMAs) were purchased from OriGene (Catalog No. CT565900). TMAs contained 50 × 1 mm cores from formalin-fixed paraffin-embedded (FFPE) samples (40 tumors and 10 normal tissues). Data for individual cases including age, gender, pathology, grade, stage, and so on can be found at http://www.origene.com/Tissue/getTissueMicroArray.aspx?id=TMA002.

Quantitative Real-Time PCR

RNA extraction from fresh frozen tissue was conducted using TRIzol as recommended by the manufacturer, followed by purification using the RNeasy Kit (Qiagen, Foster City, CA). cDNA was made using SuperScript II Reverse Transcriptase (Invitrogen, Grand Island, NY), 1× PCR buffer, $MgCl_2$ (8 mM), RNase out, and 1 to 4 μg of the appropriate total RNA. Real-time quantitative PCR assays were conducted using TaqMan probe and primers (Applied Biosystems, Grand Island, NY) for WRN (Hs00172155_m1), BLM (Hs00172060_m1), RecQL (Hs00262956_m1), RecQL4 (Hs00171627_m1), RecQL5 (Hs00188633_m1), GusB (Hs99999908_m1), and PCNA (Hs00427214_g1) along with TaqMan Universal PCR Mix (Applied Biosystems) as recommended by the manufacturer. GUSB expression was used as a loading control, and PCNA expression was used to normalize for proliferation [24–28]. The relative standard curve method was used in determining RECQ helicase expression levels. Control RNA was obtained from the following CRC cell lines: LS174T for WRN and RECQL and SW480 for BLM, RECQL4, RECQL5, and PCNA.

CpG Island Methylator Phenotype Analysis

Genomic DNA extraction from fresh frozen tissue was conducted using the DNeasy Kit (Qiagen) according to the protocol recommended by the manufacturer. Subsequently, the DNA was bisulfite converted using the Zymo Research EZ DNA methylation kit according to the manufacturer's protocol. CpG island methylator phenotype (CIMP) analysis was conducted using Methylight assays according to the protocol published by Weisenberger et al. [29], using probe primer sets and ALU-based normalization (Table W1).

The percent methylation ratio for each gene was determined for each tumor sample using the following equation: [(gene × mean value for sample)/(ALUC4 mean value for sample)]/[(gene × mean value for the methylated control)/(ALUC4 mean value for the methylated control)] * 100. A sample is scored as being CIMP positive if greater than or equal to three of five genes has a percent methylation ratio of >10.

MSI Analysis

Genomic DNA was extracted from the tissues as described. MSI status of the CHTN samples was determined from genomic DNA according to a previously published protocol [30].

RECQ Helicase Expression and Localization Using IHC

TMAs were deparaffinized and rehydrated in distilled water. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. The following antibodies and conditions were used for RECQ IHC. For WRN IHC, antigen retrieval was performed using heat-induced epitope retrieval in Epitope Retrieval 2 (Leica Biosystems, Buffalo Grove, IL). Mouse monoclonal 195C to Werner's syndrome helicase (ab62747; Abcam, Cambridge, MA) was diluted 1:100. For BLM IHC, antigen retrieval was as described for WRN. Anti-BLM antibody produced in rabbit (HPA005689; Sigma, St Louis, MO) was diluted 1:100. RECQL antigen retrieval was also as described for WRN. RECQL rabbit polyclonal antibody (sc-25547; Santa Cruz Biotechnology, Dallas, TX) was diluted 1:50. RECQL4 antigen retrieval was performed using heat-induced epitope retrieval in Epitope Retrieval 1 (Leica Biosystems). RECQL4 monoclonal antibody (M09), clone 2G8 (H00009401-M09; Abnova, Taipei City, Taiwan), was diluted 1:50. RECQL5 antigen retrieval was performed as described for WRN. Anti-RECQL5 rabbit polyclonal antibody (Sigma; HPA029971) was diluted 1:150. TMAs were incubated with primary antibody for 20 minutes in a Leica Bond XX. 3,3-Diaminobenzidine substrate was used to visualize RECQ-positive cells. TMAs were counterstained with hematoxylin. Cores were scored using the Allred scoring system, which combines two staining categories (proportion and intensity) to yield a single numerical score [31]. The Allred score ranges from 0 to 8. It is arrived upon by the addition of two scores, a proportion score and an intensity score, to reflect both the number of tumor cells stained as well as how strongly they stain. The proportion score ranges from 0 to 5 (0 = no cells stained, 1 = 1/100 cells stained, 2 = 1/10 cells stained, 3 = 1/3 cells stained, 4 = 2/3 cells stained, 5 = all cells stained), and an intensity score ranges from 0 to 3 (0 = negative, 1 = weak, 2 = intermediate, 3 = strong). Any non-epithelial cells that stained with the antibodies used were not included in this assessment.

Protein Expression Using Western Blot

Cell lysates were prepared in RIPA buffer, and the proteins were resolved using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene difluoride (PVDF) membranes and subsequently blocked in 5% non-fat dry milk in tris-buffered saline with Tween 20 (TBST). The membranes were incubated overnight with individual primary antibodies diluted 1:1000 at 4°C and then incubated for 1 hour with secondary HRP-conjugated antibodies diluted 1:5000. β-Actin was used as a protein loading control. The following antibodies were used for Western blot analysis experiments: WRN—clone 195C (Sigma); BLM—HPA005689 (Sigma); RECQL—A1107 (Santa Cruz Biotechnology); RECQL4—2814 (Cell Signaling Technology, Danvers, MA); RECQL5—HPA029971 (Sigma); PCNA—sc-7907 (Santa Cruz Biotechnology); β-actin—sc-1616 (Santa Cruz Biotechnology).

Methylation-specific PCR

Genomic DNA was extracted and bisulfite converted, and methylation-specific PCR was conducted as previously described [32]. The primer sequences used are given as follows: RECQL methyl-specific forward primer, 5′-GCGGTCCCAAAAGGGTCAGTTCGGATATCGGATAGTTAAATATCG-3′; RECQL methyl-specific reverse primer, 5′-GCGGTCCCAAAAGGGTCAGTCCGATCAACAAACGAACGTA-3′; RECQL non–methyl-specific forward primer, 5′-GCGGTCCCAAAAGGGTCAGTTGGATATTGGATAGTTAAATATTGG-3′; RECQL non–methyl-specific reverse primer, 5′-GCGGTCCCAAAAGGGTCAGTAAAAACCAATCAACAAACAAACATA-3′; RECQL5 methyl-specific forward primer, 5′-AATTAAAGGTTGTTGGTTGGTTTC-3′; RECQL5 methyl-specific reverse primer, 5′-ACTACGCGACGAATATAAAATTACG-3′; RECQL5 non–methyl-specific forward primer, 5′-AATTAAAGGTTGTTGGTTGGTTTT-3; RECQL5 non–methyl-specific reverse primer, 5′-CTACACAACAAATATAAAATTACATA-3′.

The methylation-specific PCR products were visualized using electrophoresis on a 1.5% agarose gel with ethidium bromide staining with subsequent UV transillumination using an Eagle Eye Imaging System (Stratagene, La Jolla, CA).

Statistical Analysis

As the fold change of RECQ helicase mRNA expression in tumor compared to the matched normal mucosa was determined to be non-Gaussian (KS normality and the D'Agostino and Pearson omnibus normality tests), the significance of the fold change in expression was determined using the Wilcoxon signed rank test with a theoretical median of 1. All subsequent analyses were conducted using non-parametric tests. When comparing the fold change expression of the RECQ helicases in CRC tumor to the normal colonic mucosa, a Wilcoxon signed rank test was used. Subgroup analyses of the expression of each RECQ helicase by stage were conducted using the Kruskal-Wallis test, with subsequent all-pairwise post-hoc comparisons using Mann-Whitney tests. Analysis of RECQ helicase expression by early *versus* advanced stage (stage I/II *vs* stage III/IV) was conducted using the Mann-Whitney test. Mann-Whitney and Wilcoxon signed rank tests were used to determined the statistical significance in the IHC studies as well as mRNA expression studies in CRC cell lines compared to normal colonic mucosa. All statistical tests performed were two-tailed analyses.

The fold change of RECQL5 expression was used to divide the tumor samples into two groups, those that have higher RECQL5 expression in normal mucosa compared to tumor and those that have higher RECQL5 expression in tumor compared to normal mucosa. Subsequently, the expression of the other RECQ helicases were analyzed in each of the two groups and their means compared using a Mann-Whitney test. This analysis was also conducted using the expression level of each of the RECQ helicases.

Results

Primary CRCs Have Significantly Altered Expression of RECQ Helicases

We assessed the expression of the five RECQ helicase family members in sporadic primary human CRC samples and the matched normal colon tissue. The expression of some members of the RECQ helicase family has been shown to vary throughout the cell cycle with the highest expression observed predominantly during S-phase [24,25]. As we were concerned that alterations in RECQ helicase expression in specific tumors could be a surrogate marker for proliferation rather than a cell cycle–independent occurrence in the tumor, we normalized the RECQ helicase mRNA expression data for proliferation using the expression levels of PCNA. PCNA, a cell cycle–dependent protein that accumulates in the nucleus during S-phase, can be used to normalize differences that are secondary to proliferation rates rather than basal expression [26–28]. The data without normalization for proliferation are shown in Figure W1.

Our results show that, as a group, the primary CRCs display significantly higher *BLM* and *RECQL4* mRNA expression levels compared to normal colonic mucosa ($P < .05$; Figure 1). In contrast, *RECQL* and *RECQL5* mRNA expression are lower in primary tumors compared to normal colonic mucosa ($P < .05$). There was also a significant decrease in *WRN* expression between the CRCs compared to the normal colon (Figure 1). In normal colon, expression of the RECQ helicases showed a similar degree of inter-case variability to that seen in the cancers (Figure 1). Receiver operating characteristic (ROC) analysis also showed non-identity between the expression of *WRN*, *RECQL*, *RECQL4*, and *RECQL5* in tumor *versus* normal colonic mucosa (Figure W2).

When we compared the fold change of RECQ expression in tumor *versus* normal colonic mucosa matched pairs, we observed a significantly higher level of expression of *BLM* and *RECQL4* compared to

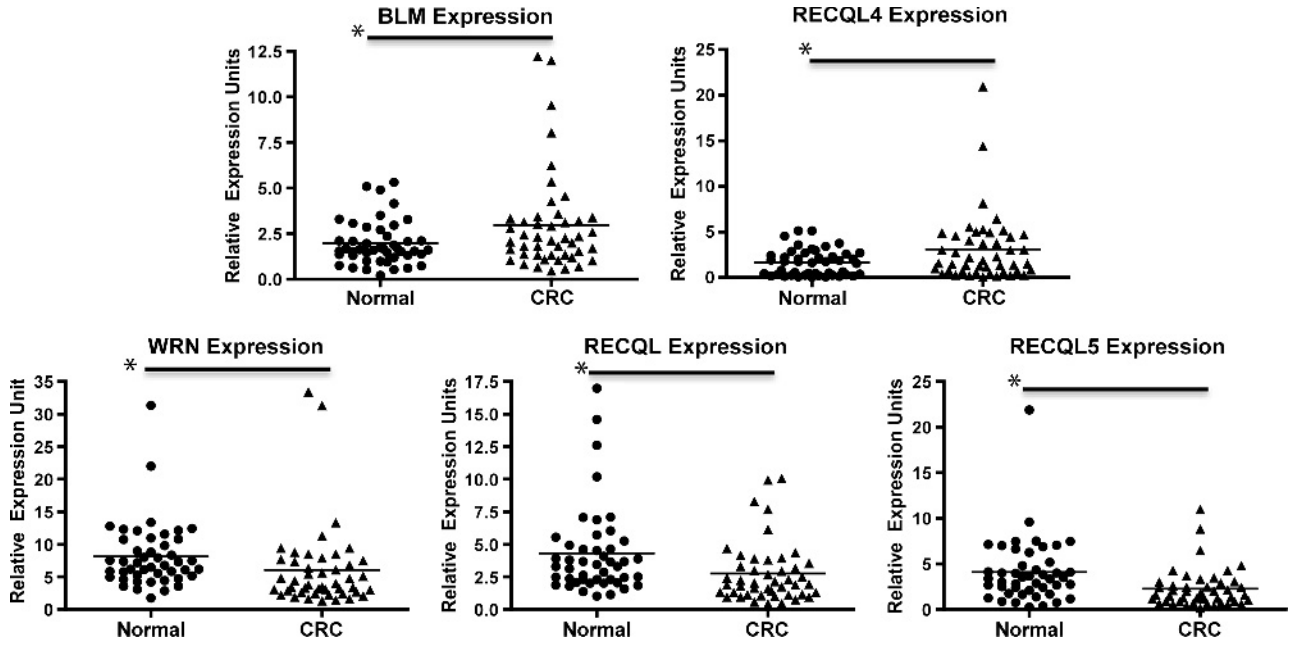


Figure 1. Expression of RECQ helicase mRNA in primary CRCs and normal colonic epithelium. The primary CRCs have higher *BLM* ($P = .0049$) and *RECQL4* ($P = .0002$) expression compared to normal colonic mucosa, whereas there is lower expression of *RECQL* ($P = .0046$), *RECQL5* ($P = .0004$), and *WRN* ($P = .0049$) in primary tumors compared to normal colonic mucosa. Asterisks indicate statistical significance between the two groups identified.

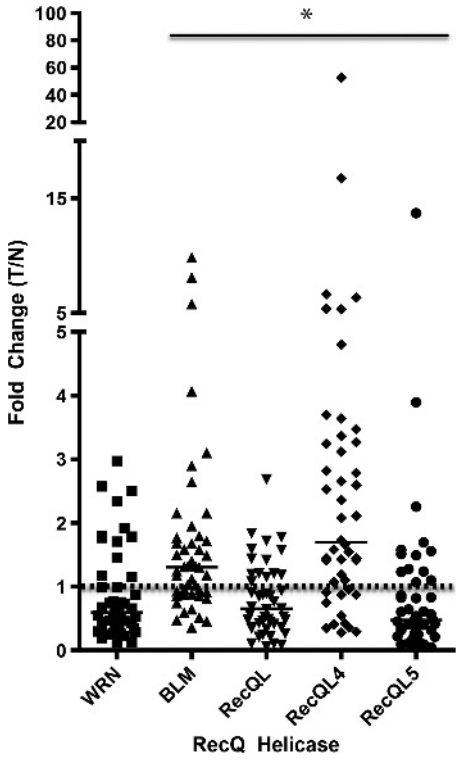


Figure 2. Fold change of RECQ helicase mRNA expression (CRC/normal colon). Solid horizontal lines indicate the median fold change for each RECQ helicase. The dashed horizontal line indicates a fold change level of 1, where the expression level of the RECQ helicase in the tumor is equal to the expression level in normal colonic mucosa. *BLM* and *RECQL4* expression is significantly higher in the CRCs compared to the matched normal colon ($P = .0011$ and $P < .0001$, respectively). *RECQL* ($P = .0103$) and *RECQL5* ($P = .0029$) expression is significantly lower in the primary CRCs compared to the matched normal colon mucosa. There is no significant difference in *WRN* mRNA expression in tumor compared to normal colonic mucosa. Asterisks indicate statistical significance in the fold change compared to the theoretical mean of 1 for the indicated helicases.

the normal mucosa (Figure 2). We also found a significantly lower level of expression of *RECQL* and *RECQL5* in the CRCs compared to matched normal mucosa (Figure 2). The tumor/normal ratios of expression are given as follows: *WRN* median = 0.5876, mean = 0.8627, SD = 0.7267 ($P = .0881$); *BLM* median = 1.303, mean = 1.795, SD = 1.836 ($P = .0011$); *RECQL* median = 0.6496, mean = 0.7973, SD = 0.568 ($P = .0103$); *RECQL4* median = 1.694, mean = 3.604, SD = 7.878 ($P < .0001$); *RECQL5* median = 0.4731, mean = 1.015, SD = 2.036 ($P = .0029$; Figure 2).

Furthermore, we observed substantial heterogeneity in the expression of *BLM* and *RECQL4* in the primary CRCs. The expression varied by 10-fold (*BLM*) and 50-fold (*RECQL4*) between CRCs (Figure 2). Our results, before normalization for proliferation, are consistent with data available on RECQ helicase expression in the Oncomine database (for RECQ helicase mRNA expression in CRC *versus* normal colonic tissue), showing an increase in the expression of all RECQ helicases. Of interest, after normalization for proliferation, we found a decrease in *WRN* and *RECQL5* expression in tumor compared to normal mucosa.

We next assessed the CRCs for correlation of expression among the RECQ helicases in CRC. We found that 3 of 46 (6.5%) cases had increased expression of all the RECQ helicases, whereas 4 of 46 (8.7%) cases had decreased expression of all five RECQ helicases compared to the respective matched normal colonic mucosa; however, this did not correlate with stage.

Characteristic Expression of RECQ Helicases in CIMP and MSI Subtypes of CRC

We also assessed RECQ helicase expression in the conventionally accepted CRC molecular subtypes (MSI and CIMP). We correlated RECQ expression level with the CIMP status of the tumors (CHTN, $N = 26$) and found that CIMP-positive tumors had high levels of *BLM* expression (tumor > normal) compared to non-CIMP tumors (Table 1). The percentage of *BLM* high tumors that displayed CIMP was 26.6%, which is greater than expected by chance. These data suggest that increased *BLM* expression in CRC may be correlated with CIMP-positive tumor status. There was no correlation between CIMP status and expression of any other RECQ helicase.

When we analyzed RECQ helicase expression level and MSI status of the primary sporadic tumors, we found that MSI CRCs (CHTN, $N = 26$) had lower *RECQL5* and *RECQL* expression than the matched normal colonic tissue (Table 2). There was no correlation between MSI status and expression of any other RECQ helicase. There was no statistically significant difference in the expression of any of the RECQ helicases between MSI and microsatellite stable (MSS) tumors.

Comparison of CRCs that Express High Levels of RECQ Helicases versus *CRCs that Express Low Levels of RECQ Helicases*

It has been shown *in vitro* that certain members of the RECQ helicase family can interact during DNA repair. Thus, we conducted an analysis of RECQ helicase expression in CRCs by dividing tumors into the following two groups: 1) those CRCs with RECQ helicase expression greater than normal mucosa and 2) those CRCs with RECQ helicase expression less than normal mucosa. This was done to determine if distinct populations of CRCs with high or low expression of the RECQ helicases could be identified on the basis of expression of any one of the RECQ helicases. To determine if we could identify an association in the expression level of specific RECQ helicases, we assessed the correlation between the expression of RECQ helicases in a pairwise fashion.

We found that grouping cases by the expression level of *RECQL5* as either high (tumor > normal) or low expression (tumor < normal) allowed us to predict similarly high or low expression levels of *WRN* and *RECQL*. Grouping of cases by *RECQL5* high or low expression

Table 1. CIMP Status of CRC Corresponds with Increased *BLM* Expression.

BLM Expression	Non-CIMP	CIMP	Total Cases	Percent CIMP	Group Mean	SD
T < N	11	0	11	0	0.75	0.22
T > N	11	4	15	26.6	2.62	2.59
All cases	22	4	26	15.3		

When *BLM* expression is used to cluster cases into CRCs with *BLM* expression less in tumor compared to normal and CRCs with *BLM* expression greater in tumor compared to normal, we identified two distinct subgroups of CRCs ($P < .05$). Although clustering of cases by *BLM* expression does not predict differences in expression of any of the other four RecQ helicases, it does correlate with CIMP status. CIMP-positive tumors were observed only in cases where the expression of *BLM* was greater in the tumors compared to the normal colon. These results suggest that CIMP CRCs tend to correlate with higher *BLM* expression in the tumor compared to matched normal mucosa and not cases where *BLM* expression is decreased in the tumor compared to matched normal mucosa.

Table 2. MSI Tumors Have Low Expression of *RECQL* and *RECQL5*.

Gene Expression	MSS	MSI	Total Cases	Percent MSI	Group Mean	SD
RECQL, T < N	12	6	18	33.3	0.50	0.30
RECQL, T > N	8	0	8	0	1.71	0.45
RECQL5, T < N	11	6	17	35.3	0.38	0.25
RECQL5, T > N	9	0	9	0	2.84	4.09
All cases	20	6	26	23.1		

When either *RECQL* or *RECQL5* expression was used to cluster cases into those that expressed the gene of interest less than the matched normal mucosa or those that expressed the gene more than the matched normal mucosa, we were able to identify two distinct subgroups ($P < .05$). MSI CRCs were observed only in cases where the expression of *RECQL* and *RECQL5* were less in the CRC compared to the normal colon. These results suggest that MSI cases correlate with low *RECQL* and *RECQL5* expression in the tumor compared to the matched normal mucosa. MSS, microsatellite stable (MSI negative).

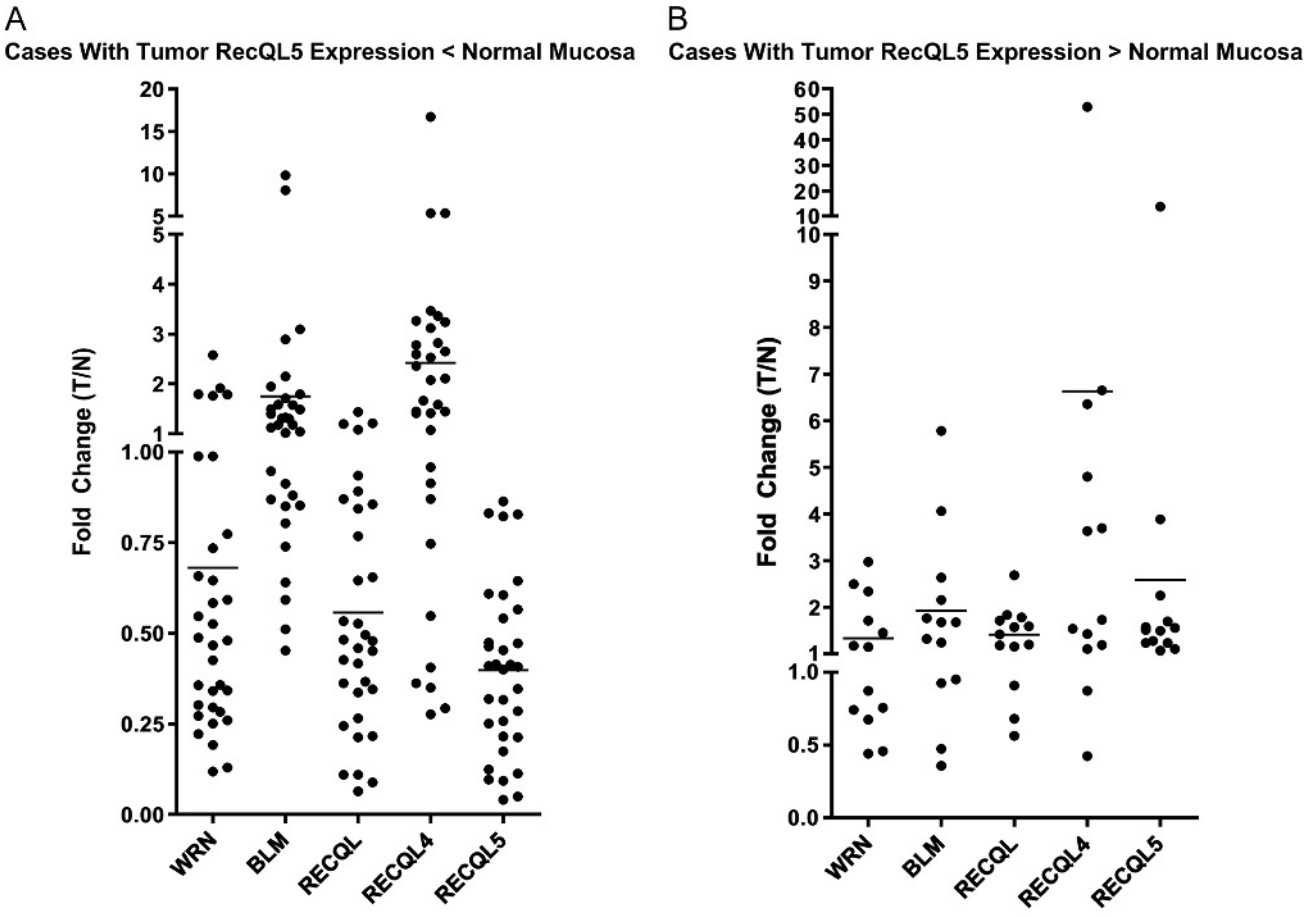
identified distinct populations of CRCs with regard to *RECQL5*, *WRN*, and *RECQL* expression ($P < .05$; Figure 3). Of the tumors that expressed *RECQL5* < normal mucosa, 88% also had low levels (T < N) of *RECQL* expression, and 85% of those tumors had low levels (T < N) of *WRN* expression (Figure 3).

We also found that grouping cases by low expression levels of either *RECQL* or *WRN* was able to predict low levels of *RECQL5*, confirming the relationship seen when the cases were grouped using *RECQL5* expression. Grouping cases by expression of *RECQL4*, however, did not predict differences in expression of the other RECQ helicases.

When *BLM* expression was used to group tumors into high (tumor > normal) and low (normal < tumor) *BLM*-expressing groups, we were not able to predict expression of any of the other RECQ helicases. However, CIMP-positive cases were only found among the *BLM* high tumors and were not found in BLM low tumors (Table 1).

RECQ Expression Analysis by Stage

Subgroup analyses of the mRNA expression of each RECQ helicase by stage showed a statistically significant difference in the expression of *WRN* in the CRCs compared to normal mucosa by stage ($P = .0498$), with the highest average value seen in stage II CRCs (Figure W3). However, after all-pairwise post-hoc comparisons, there was not enough power to detect differences in *WRN* expression among



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		WRN			BLM			RecQL			RecQL4			RecQL5		
RecQL5	Total Cases	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value	Mean	SD	p value
T < N	33	0.68	0.60	< 0.05	1.74	1.97	0.306	0.56	0.35	< 0.05	2.41	2.90	0.205	0.40	0.23	< 0.05
T > N	13	1.33	0.83		1.93	1.51		1.41	0.56		6.63	14.02		2.58	3.42	

Figure 3. Classifying CRC cases by *RECQL5* expression can predict the expression levels of *WRN* and *RECQL*. (A) Of the tumors that expressed *RECQL5* < normal mucosa, 88% also had low levels (T < N) of *RECQL* expression. (B) Of tumors that expressed *RECQL5* > normal mucosa, 85% had low levels (T < N) of *WRN* expression. (C) Grouping cases by *RECQL5* expression T < N or T > N showed statistical significance in expression of *RECQL5*, *RECQL*, and *WRN* ($P < .05$).

the stages. No significant differences in the expression of *BLM* (P = .2193), *RECQL* (P = .5992), *RECQL4* (P = .4077), and *RECQL5* (P = .3889) among CRC stages I to IV were observed. The significance of these results is unclear given the low level of expression of WRN in the colon and the poor correlation of WRN mRNA expression with WRN protein expression that we have observed (see below).

When we compared the expression levels in localized disease *versus* advanced disease (stages I and II *vs* stages III and IV), there were no significant differences in RECQ helicase expression (P > .05). As described in the next section, the same analysis was conducted using Allred scores determined from IHC staining of RECQ helicases in archival CRC cases. The results from the IHC analysis also show no significant difference in protein expression for any of the RECQ helicases across stage, WRN (P = .5709), BLM (P = .3644), RECQL4 (P = .5451), RECQL (P = .1922), or RECQL5 (P = .5604; Figure W3).

Nuclear Localization of RECQ Helicase in Primary CRC Tumors

IHC studies were conducted to determine the localization of the RECQ helicases in CRC tumor tissue and normal colonic mucosa. For all RECQ helicases, IHC analysis consistently localized the helicases to the nucleus (Figure 4). However, RECQL5 was also observed in the cytoplasm as well as in the nucleus. Analysis of Allred scores for each of the RECQ helicases showed decreased expression of RECQL5 in tumors compared to normal mucosa (P < .05), consistent with the *RECQL5* mRNA levels we observed (Figure 4). WRN IHC revealed an increase in expression in tumors compared to normal colon mucosa (P < .05), suggesting that protein expression levels and mRNA expression levels for WRN do not correlate well, consistent with data obtained from CRC cell lines (Figure 5). The other RECQ helicases also show statistically significant differences in expression between tumor and normal tissue (P < .05; Figure 5); however, given the small differences in the Allred score between tumor and normal tissue, we believe that this is unlikely to be clinically significant. When the intensity score component of the Allred staining score was analyzed separately from the proportion score component, we saw a significant difference in the reduction of RECQL staining intensity and a significant increase in RECQL4 proportion of staining in tumor compared to normal mucosa (P = .0226 and P = .0002, respectively), consistent with the results obtained for *RECQL* and *RECQL4* mRNA expression.

CRC Cell Lines Show Expression of RECQ Helicases in a Pattern Similar to Primary CRCs

Given the significant alterations in RECQ helicase expression we observed in primary CRCs, we assessed the expression of RECQ helicases in a panel of CRC cell lines to determine the feasibility of using cell lines to study the functional consequences of alterations in the expression levels of the RECQ helicases. We studied CRC cell lines derived from the three major molecular subtypes of CRC, MSS (or CIN) cancers (SW480, V400, V411, AAC1/SB10, and HT29), MSI cancers (HCT116, LoVo, and LS174T), and CIMP cancers (RKO, SW48, and FET). Similar to the pattern of RECQ helicase expression seen in primary CRCs, the cell lines had higher mRNA expression levels of *BLM* and *RECQL4* than *RECQL*, *RECQL5*, and *WRN*. This was true regardless of the molecular subgroup of the CRC cell line. *WRN* had the lowest average mRNA expression level of all the RECQ helicase family members (Figure 5*A*). We observed low expression of *RECQL* (P < .001) and significantly high expression of *RECQL4* (P = .017) in CRC cell lines when compared to normal colonic mucosa (Figure 5*B*), which is consistent with the expression levels we observed in the primary CRCs.

We subsequently assessed RECQ helicase protein expression by immunoblotting protein lysates from these lines (Figure 5*C*). We found that WRN and RECQL5 protein levels correlated poorly with mRNA levels but that BLM, RECQL, and RECQL4 showed similar patterns of mRNA and protein expression. Interestingly, we found considerable variability among the CRC cell lines, even after normalization for proliferation, of both mRNA and protein expression for BLM and RECQL4. There was up to approximately three-fold difference in expression levels across cell lines (Figure 5, *B* and *C*).

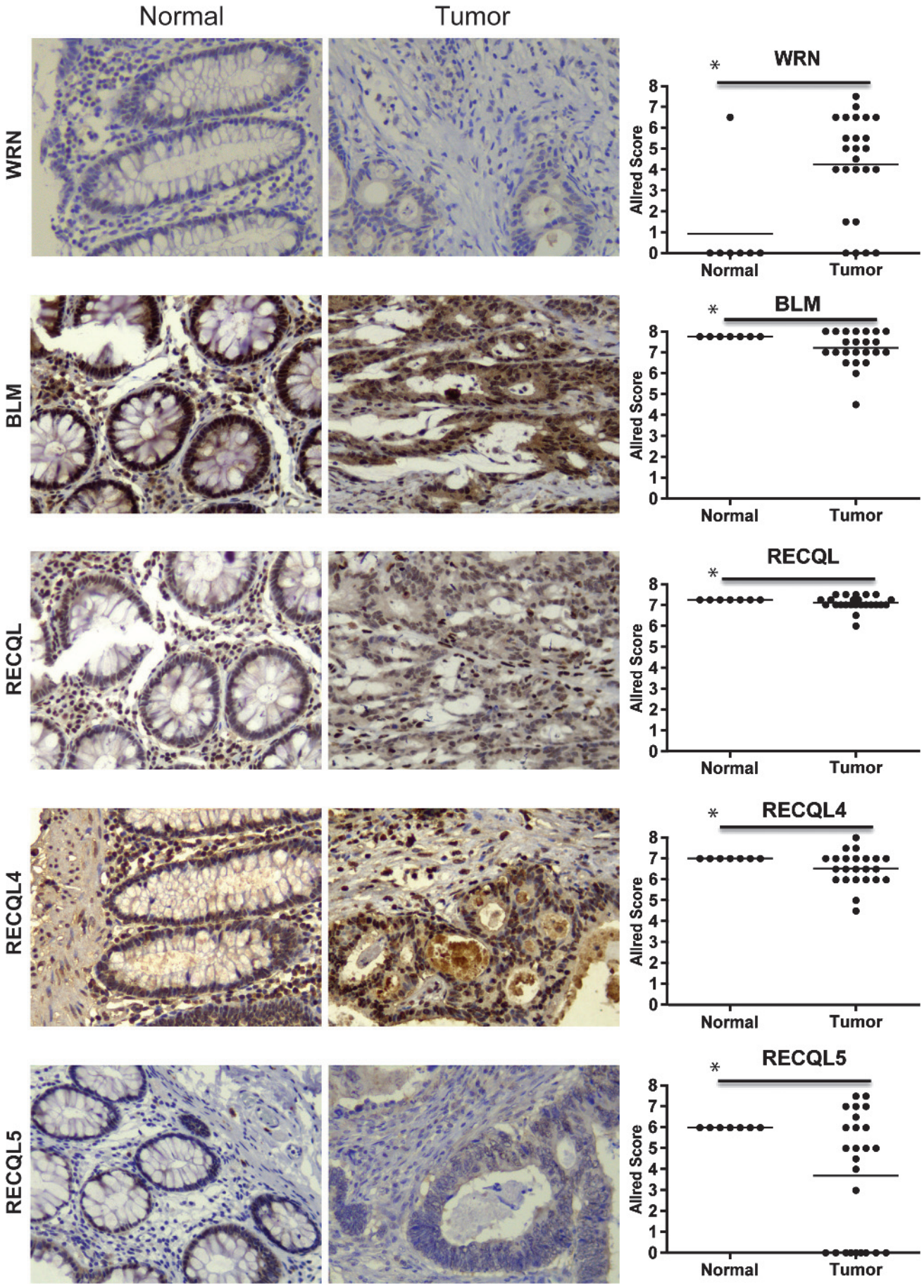
Promoter Methylation Status of RECQL and RECQL5

To gain further understanding about the decreased expression of RECQL and RECQL5 seen in CRCs, we assessed the promoter methylation status of both *RECQL* and *RECQL5* in normal colonic mucosa as well as CRC tumors. Our results show the absence of methylation in both the *RECQL* (N = 0/7) and *RECQL5* (N = 0/8) promoters in normal colonic mucosa. We also found that 14% of CRCs have an aberrantly methylated *RECQL5* promoter (N = 3/21), whereas 0% of CRCs have a methylated *RECQL* promoter (N = 0/16). Thus, the aberrant methylation of the promoters of *RECQL* and *RECL5* does not seem to be a significant mechanism for silencing the expression of these genes.

Discussion

The RECQ helicases are a family of enzymes that maintain genomic stability through their function in DNA processing as helicases and exonucleases. They participate in DNA repair as members of DNA repair complexes [33]. They have been shown to be involved in single-strand break and double-strand break repair [34] as well as in the repair of topoisomerase I–mediated DNA damage induced by

Figure 4. IHC staining shows localization of RECQ helicases in normal colonic mucosa and CRC. All of the RECQ helicases were detected in the nucleus in both normal colonic mucosa and CRCs. Allred scores for each of the RECQ helicases from IHC of TMAs are also presented. Low expression of RECQL5 in tumors compared to normal mucosa can be seen (P = .0031, Wilcoxon signed rank test), which is consistent with the results obtained from the RECQL5 qRT-PCR assays. WRN showed higher expression in tumors compared to normal colon mucosa (P = .0128, Mann-Whitney), suggesting that immunostaining scores and mRNA expression levels of WRN do not correlate. The other RECQ helicases also show statistically significant differences in expression between tumor and normal tissue (BLM, P = .0046, Wilcoxon signed rank test; RECQL, P = .0287, Wilcoxon signed rank test; RECQL4, P = .0103, Wilcoxon signed rank test); however, this difference likely does not translate to clinical significance. Allred scores for the selected matched tumor normal images chosen to show localization are given as follows: WRN: N = 7, T = 0; BLM: N = 7.75, T = 7; RECQL: N = 7.25, T = 6; RECQL4: N = 7, T = 8; RECQL5: N = 6 (cytoplasmic 2), T = 0 (cytoplasmic 2). All images were photographed at ×20. Asterisks indicate statistical significance between the two groups identified.


Normal
Tumor
WRN
BLM
RECQL
RECQL4
RECQL5
Allred Score
Normal
Tumor
*

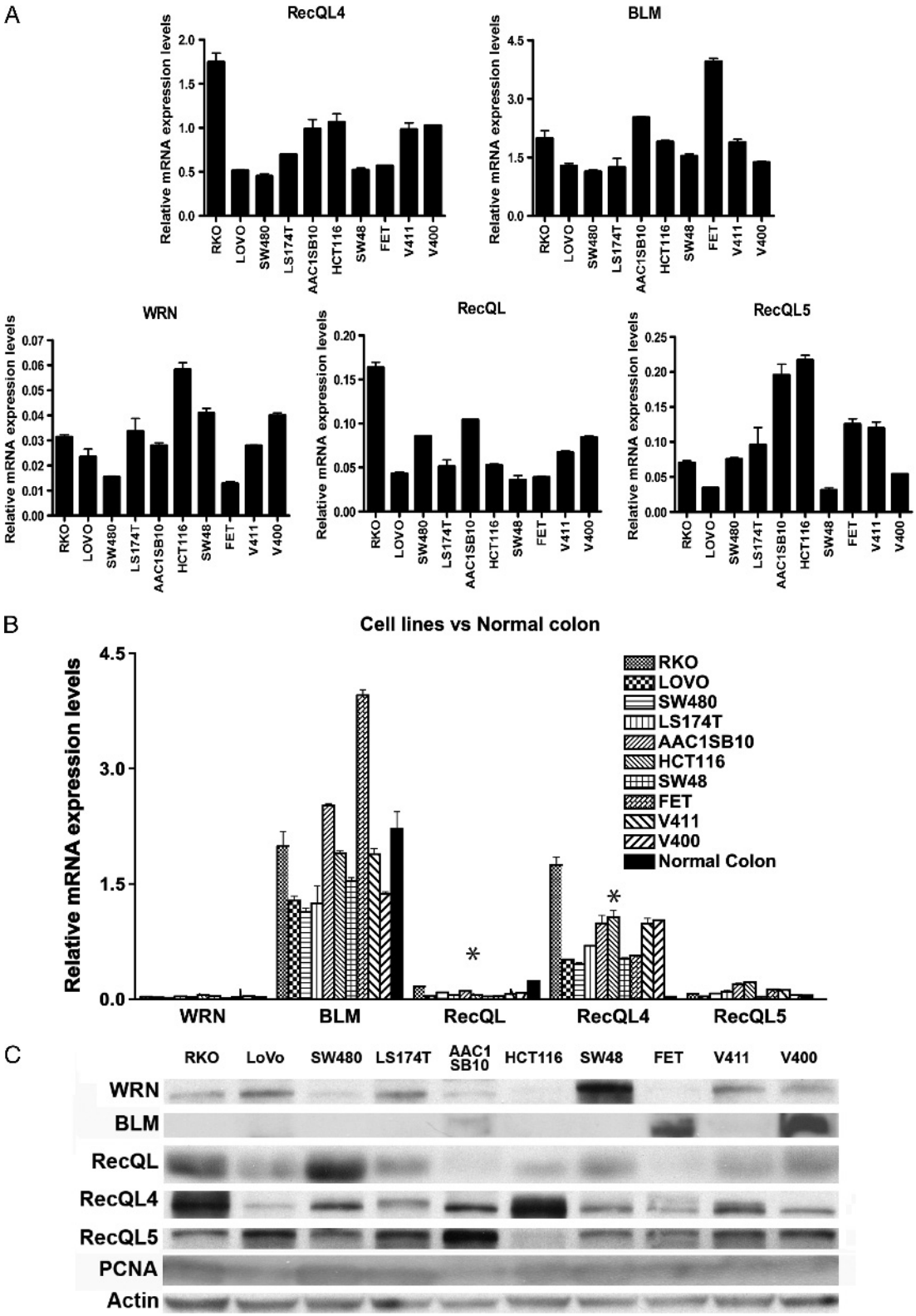


Figure 5. RECQ helicase expression in CRC cell lines. (A) RECQ helicase mRNA expression was assessed in CRC lines representing the three major molecular subtypes of CRC, MSS (or CIN) cancers (SW480, V400, V411, AAC1/SB10, and HT29), microsatellite unstable (MSI) cancers (HCT116, LoVo, and LS174T), and CIMP cancers (RKO, SW48, and FET). Expression levels of *BLM* and *RECQL4* mRNA were higher than those of *RECQL, RECQL5*, and *WRN*, regardless of molecular subgroup. (B) RECQ helicase mRNA expression compared to normal colonic mucosa for CRC cell lines. *RECQL4* expression is significantly higher in the cell lines compared to the normal colonic mucosa ($P = .002$), whereas RECQL expression is significantly decreased ($P < .002$). Asterisks indicate statistical significance between the average expression of the indicated helicase in CRC cell line compared to normal colonic mucosa. (C) RECQ helicase Western blot results for CRC cell lines. PCNA and actin were both run as normalization controls in light of the known correlation of RECQ helicase expression with the phase of the cell cycle.

camptothecin derivatives such as irinotecan [16]. The loss of function of three of the RECQ helicases, WRN, BLM, and RECQL4, results in human syndromes characterized by a predisposition to cancer, including CRC [33]. In addition, *in vitro* studies demonstrate that these helicases are important in protecting genomic DNA from genotoxic stress [15,18,24,34–37]. Therefore, we postulated that the expression of the RECQ helicases is altered in CRC and that the aberrant expression of these RECQ helicases may have a role in CRC pathogenesis and in mediating the response of CRC cells to DNA-damaging chemotherapeutic agents. Our results confirm that the expression of RECQ helicases in primary CRC tumors is altered compared to normal colonic mucosa. It is plausible that these changes may be biologically relevant in light of the fact that most RECQ helicases act in protein complexes and even small changes in expression level could disrupt the stoichiometry of these complexes and their function. However, the biologic significance of these changes has yet to be determined. Our current findings support conducting further studies of these helicases to determine if they could be predictive biomarkers or therapeutic targets in CRC.

Our results are consistent with previous findings that RECQL5 may have tumor suppressor effects in CRC [18,19]. We found that *RECQL* and *RECQL5* are expressed at statistically significant decreased levels in CRC when compared to matched normal colonic mucosa (Figure 2). Although loss of function of RECQL and RECQL5 have not been clearly linked to human disease, there is evidence from studies of mouse models as well as *in vitro* systems that loss of function of these helicases would likely lead to disease given their role in the maintenance of genomic stability [35,36,38–41]. RECQL5 is implicated in DNA replication, transcription, and repair processes, and RECQL5 deficiency is thought to contribute to genomic instability [18,42]. Our data also show that MSI tumors uniformly have decreased RECQL and RECQL5 expression (Table 2). These data suggest that RECQL5 and RECQL may have a role in mediating the degree of MSI seen in MSI CRC warranting further investigation. Furthermore, RECQL5-deficient $Apc^{Min/wt}$ mice have been shown to have an increase in the incidence of tumors formed in the colon. As RECQL5 is highly conserved between mice and humans, these results are consistent with a role for RECQL5 as a tumor suppressor in human CRC [19]. Our data establish the overall decreased expression of both *RECQL* and *RECQL5* in CRCs and are consistent with previous studies suggesting that a decrease in *RECQL5* expression in tumors would contribute to the pathogenesis of CRC [18,19,36,37].

We also found that grouping CRC tumors by *RECQL5* expression status (high or low) results in two classes of CRC that are distinct not only in their expression of *RECQL5* but also in their expression of *WRN* and *RECQL* (Figure 3). RECQL5 low (tumor < normal) CRCs tend to also have low WRN and RECQL expression. These data suggest that there may be a biologic link among these three RECQ helicases. Recently, it has been shown that RECQL5 is important for cell survival after treatment with camptothecin, a topoisomerase inhibitor [20,42]. Therefore, tumors with decreased expression of *RECQL5* compared to normal colonic mucosa, with similarly decreased expression of *WRN* and *RECQL*, may represent a population of cells that are particularly susceptible to topoisomerase I inhibitors such as irinotecan. Further studies will help provide insight into this possibility.

In contrast to *RECQL* and *RECQL5*, we found that *RECQL4* and *BLM* expression are significantly increased in CRCs when compared to matched normal colonic mucosa (Figure 2). An increase in the expression of these two RECQ helicases may confer a survival advantage to CRC cells and thereby contribute to the pathogenesis of CRC. RECQL4 and BLM have been shown to have coordinated activities and to cooperate in repairing DNA damage caused by ionizing radiation [24]. Our data are consistent with the possibility that increased expression of BLM and RECQL4 promote the ability of CRC cells to tolerate genotoxic stress. Functional studies are now needed to assess this possibility.

We found increased expression of BLM in primary CRCs despite its role as a tumor suppressor gene in Bloom syndrome and mouse intestinal cancer [43,44]. Increased BLM expression in established CRCs may provide a survival advantage for tumor cells, as it has been shown that BLM can facilitate telomere replication [45,46]. BLM has been co-localized with POT1, TRF1, and TRF2 (proteins that regulate telomere stability and length) in telomerase-deficient, alternative lengthening of telomeres–positive cells. When BLM is depleted in these cells, rapid telomere shortening was shown to occur [45,47]. Given previous data showing that loss of BLM leads to increased susceptibility of cells to killing with DNA-damaging agents [23,34], CRCs that showed an increase in BLM expression compared to normal colonic mucosa may be a subset of cancers that have decreased sensitivity to DNA-damaging agents. Our data showing that CIMP CRCs all have high (tumor > normal) *BLM* expression may have implications with regard to the effect of chemotherapy on CIMP CRCs compared to non-CIMP CRCs.

We also observed significant overexpression of *RECQL4* in the CRCs. Recent studies show that RECQL4 expression is also increased in metastatic prostate cancer cell lines [48] as well as in sporadic osteoblastomas [49]. Loss of RECQL4 activity leads to an accumulation of double-strand breaks, and knockdown of RECQL4 decreases both tumorigenicity and invasiveness [48]. Therefore, increased expression of RECQL4 may likewise be important in the pathogenesis of CRC, conferring a survival advantage to the tumor. The data show that *BLM* and *RECQL4* are the only two RECQ helicases that have increased expression. This is consistent with the co-localization of RECQL4 and BLM during S-phase and their reported coordinated activity [24]. Consistent with previous cell culture studies, our IHC studies show that BLM and RECQL4 are indeed localized to the nucleus (Figure 4). The expression of both *BLM* and *RECQL4* widely range in fold change values, making them good candidates to investigate for a role as predictive biomarkers in CRC.

Our studies showing abnormal expression of the RECQ helicases in CRC suggest that the RECQ helicase genes may also be targets for mutations. Therefore, as an adjunct, we assessed the status of reported RECQ helicase mutations in CRCs in The Cancer Genome Atlas (TCGA) database (cancergenome.nih.gov/; 20/224 cases) and determined the predicted functional consequence of these mutations (Tables W2–W5). We found that there are no reported mutations in *RECQL4*, whereas there are reported mutations in *BLM* (4.5%), *WRN* (4.0%), *RECQL* (1.8%), and *RECQL5* (2.7%). Of the reported mutations, approximately half are predicted to disrupt the function of the gene. Thus, mutational inactivation of the RECQ helicases appears to be a second mechanism through which potential tumor suppressor functions of the RECQ helicases may be disrupted. Furthermore, the lack of reported mutations in *RECQL4* and the increased expression of RECQL4 seen in CRCs suggest that this enzyme may be important for tumorigenesis.

Potential mechanisms that control RECQ helicase expression include epigenetic alterations, such as promoter methylation. Our results show that aberrant promoter methylation of *RECQL5* occurs

in 14% of CRC tumors, suggesting that promoter methylation is responsible for the transcriptional repression of RECQL5 of only a small subset of CRCs. However, aberrant DNA methylation does not appear to be the mechanism responsible for the down-regulation of RECQL in CRCs, as we did not detect *RECQL* promoter methylation in CRCs. Other possible regulatory mechanisms for RECQ helicases include alterations in transcription factors that enhance or suppress gene expression and post-transcriptional processing of the mRNA. There is evidence that RECQL5 is regulated in part by transcription factor binding with a zinc-binding motif, which is highly conserved among RECQ helicases [50]. There are also data showing that RECQL5 interacts with RAD51 and that this interaction is critical for specific RECQL5 function [51]. Furthermore, E2F binding sites have been identified in RECQ helicase promoters to which both E2F and Rb can bind and recruit an Rb family repressor complex [52]. The precise mechanism by which the RECQ helicases are regulated is not fully understood; however, it is clear that this family of proteins have altered expression in CRCs.

An important technical issue related to our studies is the discrepancy we observed between the mRNA and protein expression of certain RECQ helicases in the primary tumors. In our hands, IHC stains for RECQ expression proved to be less precise in the quantitation of RECQ expression differences compared to qRT-PCR, with many of the Allred scores clustering at high levels for RecQL, RecQL4, and BLM. This is likely because of the more precise quantitative nature of the qRT-PCR assay compared to the more qualitative nature of IHC. The lack of correlation in WRN IHC when compared to mRNA expression in primary tissues was less surprising, as this was consistent with WRN protein expression compared to mRNA expression in CRC cell lines assayed by Western blot analysis. The data suggest that *WRN* mRNA levels indeed correlate poorly with protein levels. The differences seen in expression of RECQL5 in tumor compared to normal colonic mucosa were consistent at both the mRNA and protein levels, suggesting that these differences are the most robust and the results from the RT-PCR assays are comparable to the IHC studies.

In conclusion, we have shown that there are significant differences among CRCs with regard to the expression of the RecQ helicases as well as differences in the expression of RecQ helicases between CRCs and normal colonic tissues. On the basis of the known function of these helicases, the altered expression of RECQL, RECQL5, RECQL4, and BLM may affect the formation of CRC as well as the response of CRCs to chemotherapy. We also observed characteristic expression patterns of RECQ helicases in the different molecular subtypes of CRC, suggesting that the expression of these DNA repair enzymes may prove to help classify tumor behavior with regard to response to therapy. The studies presented here establish the altered expression profile of RECQ helicases in primary CRCs and provide a strong rationale for further studies of these helicases as predictive biomarkers or therapeutic targets in CRC.

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Table W1. Probe and Primer Sequences Used in CIMP Analyses of the Colorectal Tumors Using Methylight according to the Protocol Published by Weisenberger et al..

Gene	Probe	Forward Primer	Reverse Primer
Calcium channel, voltage-dependent, alpha 1G subunit	6FAM-AAATAACGCCGAATC CGACAACCGA-BHQ-1	TTTTTTCGTTTCGCGTTTAGGT	CTCGAAACGACTTCGCCG
Insulin-like growth factor 2 (somatomedin A)	6FAM-CCCTCTACCGTCGCG AACCCGA-BHQ-1	GAGCGGTTTCGGTGTCGTTA	CCAACTCGATTTAAACCGACG
Neurogenin 1	6FAM-CGATAACGACCTCCC GCGAACATAAA-BHQ-1	CGTGTAGCGTTCGGGTATTTGTA	CGATAATTACGAACACACTCCGAAT
Runt-related transcription factor 3	6FAM-CGCACGAACTCGCC TACGTAATCCG-BHQ-1	CGTTCGATGGTGGACGTGT	GACGAACAACGTCTTATTACAACGC
Suppressor of cytokine signaling 1	6FAM-ACAATTCCGCTAAC GACTATCGCGCA-BHQ-1	GCGTCGAGTTCGTGGGTATTT	CCGAAACCATCTTCACGCTAA
ALU	6FAM-CCTACCTTAACCTCCC-MGBNFQ	GGTTAGGTATAGTGGTTTATATT TGTAATTTTAGTA	ATTAACTAAACTAATCTTAAACTCCT AACCTCA

All sequences are provided 5′ to 3′.

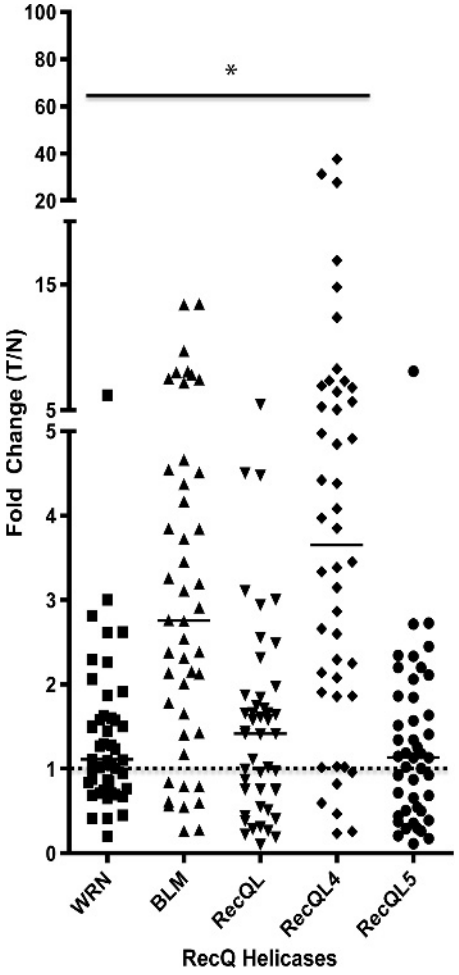

Figure W1. Fold change of RECQ helicase mRNA expression without normalization for proliferation using *PCNA* mRNA expression (CRC/normal colon). Solid horizontal lines indicate the median fold change for each RECQ helicase. The dashed horizontal line indicates a fold change level of 1, where the expression level of the RECQ helicase in the tumor is equal to the expression level in normal colonic mucosa. *WRN* ($P = .0259$), *BLM* ($P < .0001$), *RECQL* ($P < .0183$), and *RECQL4* ($P < .001$) expression levels are significantly increased in tumor compared to normal mucosa. There is no significant difference in *RECQL5* mRNA expression in tumor compared to normal colonic mucosa ($P = .182$). Asterisks indicate statistical significance in the fold change compared to the theoretical mean of 1 for the indicated helicases.

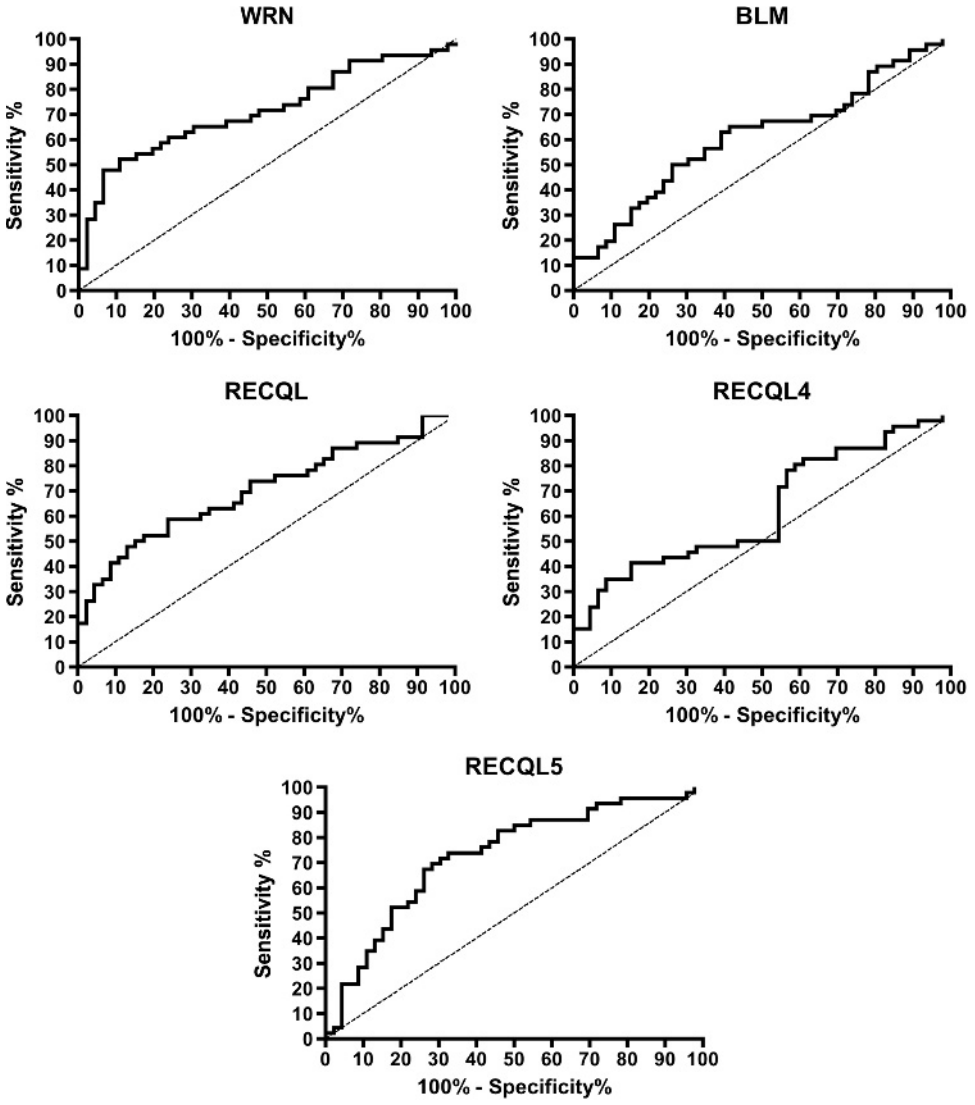

Figure W2. ROC curves for RECQ helicase expression in CRC and normal colonic mucosa. The area under the curve and 95% confidence intervals for each ROC analysis are given as follows: WRN, 0.7136 (0.6067-0.8205); BLM, 0.6101 (0.4940-0.7262); RECQL, 0.6994 (0.5920-0.8069); RECQL4 (0.5103-0.7402); RECQL5 (0.6236-0.8329). Dashed line is the line of identity.

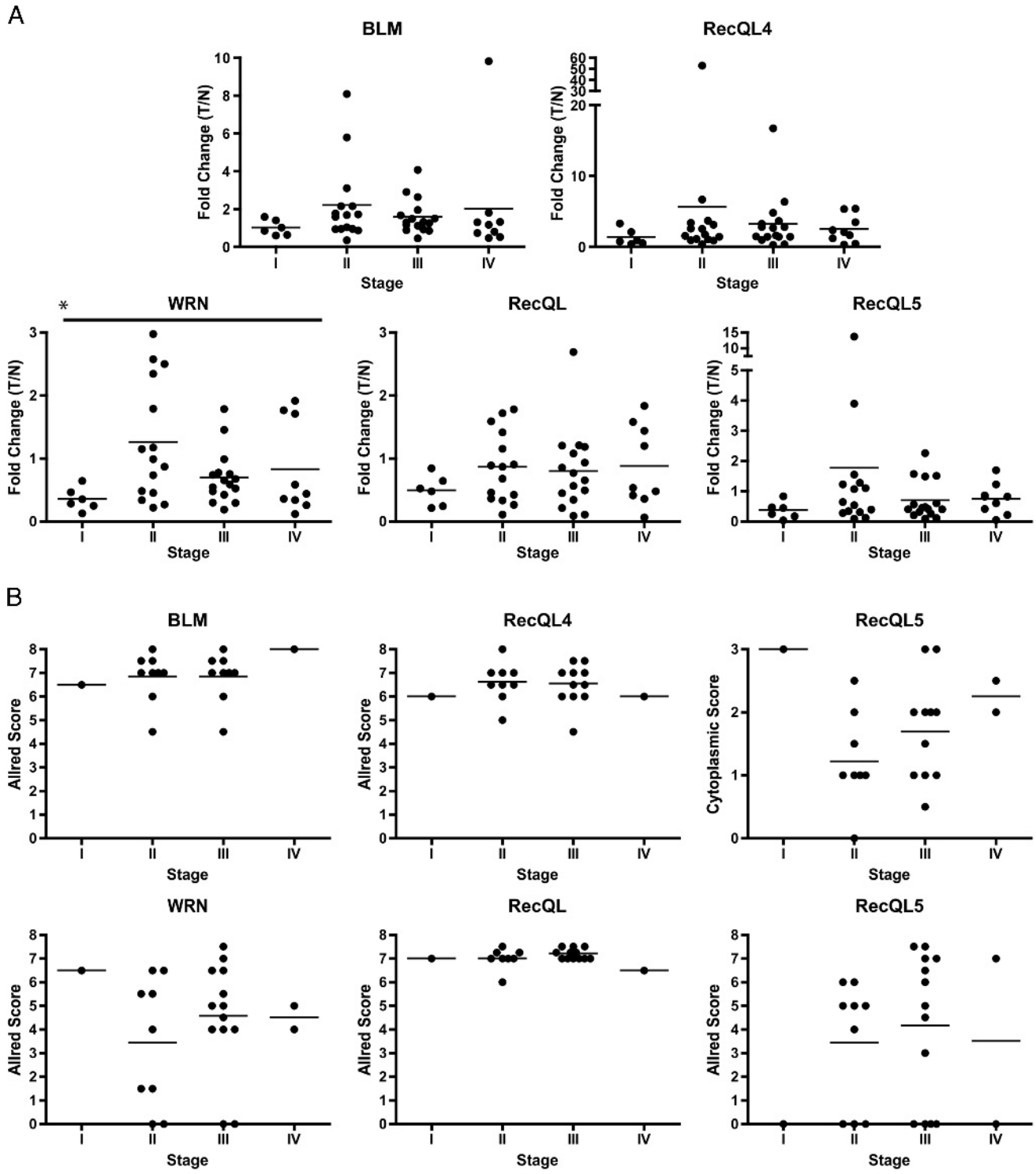

Figure W3. Analysis of RECQ helicase expression by CRC stage. (A) RECQ helicase mRNA expression. There is a significant difference in fold change of *WRN* expression in the CRCs compared to normal mucosa by stage ($P = .0498$); however, after all-pairwise post-hoc comparisons, there was not enough power to detect differences among the stages. The highest median expression is seen in stage II CRC. Higher expression levels of *WRN* in stage II compared to lower expression levels of *WRN* in the other tumor stages (compared to matched normal mucosa) can be appreciated. However, no statistical significance is seen in the expression of *BLM* ($P = .2193$), *RECQL4* ($P = .4077$), *RECQL* ($P = .5992$), or *RECQL5* ($P = .3889$) in the CRCs compared to matched normal tissue in any of the stages. (B) RECQ helicase IHC. On the basis of Allred scores, there is no statistically significant difference in expression of the RECQ helicases in CRCs across stages, WRN ($P = .5709$), BLM ($P = .3644$), RECQL4 ($P = .5451$), RECQL ($P = .1922$), or RECQL5 ($P = .5604$).

Table W2. Somatic BLM Mutations Identified by TCGA in CRCs and Their Predicted Impact.

BLM AA Mutation	Mutation Type	Mutation Assessor	PolyPhen-2	Exome Variant
L647I	Missense	Low	Benign	No
A964T	Missense	Low	Benign	No
F1087C	Missense	Low	Benign	No
S1252F	Missense	Low	Possibly damaging	No
S147Y	Missense	Medium	Probably damaging	No
Y784C	Missense	Medium	Possibly damaging	No
R1144I	Missense	Medium	Possibly damaging	No
A703V	Missense	High	Probably damaging	No
Y764C	Missense	High	Probably damaging	No
HPfs (3518)	Frameshift	–	–	–
HPfs (3811)	Frameshift	–	–	–

These predictions were obtained using Mutation Assessor v.2 and PolyPhen-2 HumVar algorithm. The following web resources were used: Mutation Assessor, http://mutationassessor.org; PolyPhen-2, http://genetics.bwh.harvard.edu/pph2; Exome Variant, http://evs.gs.washington.edu/EVS.

Table W3. Somatic WRN Mutations Identified by TCGA in CRCs and Their Predicted Impact.

BLM AA Mutation	Mutation Type	Mutation Assessor	PolyPhen-2	Exome Variant
L647I	Missense	Low	Benign	No
A964T	Missense	Low	Benign	No
F1087C	Missense	Low	Benign	No
S1252F	Missense	Low	Possibly damaging	No
S147Y	Missense	Medium	Probably damaging	No
Y784C	Missense	Medium	Possibly damaging	No
R1144I	Missense	Medium	Possibly damaging	No
A703V	Missense	High	Probably damaging	No
Y764C	Missense	High	Probably damaging	No
HPfs (3518)	Frameshift	–	–	–
HPfs (3811)	Frameshift	–	–	–

These predictions were obtained using Mutation Assessor v.2 and PolyPhen-2 HumVar algorithm. The following web resources were used: Mutation Assessor, http://mutationassessor.org; PolyPhen-2, http://genetics.bwh.harvard.edu/pph2; Exome Variant, http://evs.gs.washington.edu/EVS.

Table W4. Somatic RECQL Mutations Identified by TCGA in CRCs and Their Predicted Impact.

RECQL AA Mutation	Mutation Type	Mutation Assessor	Polyphen-2	Exome Variant
A248T	Missense	Neutral	Benign	No
N397K	Missense	Medium	Probably damaging	No
S568L	Missense	Medium	Probably damaging	No
V41*	Frameshift	–	–	–

These predictions were obtained using Mutation Assessor v.2 and PolyPhen-2 HumVar algorithm. The following web resources were used: Mutation Assessor, http://mutationassessor.org; PolyPhen-2, http://genetics.bwh.harvard.edu/pph2; Exome Variant, http://evs.gs.washington.edu/EVS.
*Signifies deletion (caused by the frameshift mutation).

Table W5. Somatic RECQL5 Mutations Identified by TCGA in CRCs and Their Predicted Impact.

RecQL5 AA Mutation	Mutation Type	Mutation Assessor	Polyphen-2	Exome Variant
E573D	Missense	Neutral	Benign	No
L556I	Missense	Low	Benign	No
C832Y	Missense	Low	Benign	No
E980K	Missense	Medium	Possibly damaging	No
G71C	Missense	Medium	Probably damaging	No
T316I	Missense	High	Probably damaging	No

These predictions were obtained using Mutation Assessor v.2 and PolyPhen-2 HumVar algorithm. The following web resources were used: Mutation Assessor, http://mutationassessor.org; PolyPhen-2, http://genetics.bwh.harvard.edu/pph2; Exome Variant, http://evs.gs.washington.edu/EVS.