Functional effects of the *MLH1*-93G>A polymorphism on *MLH1/EPM2AIP1* promoter activity

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Abstract. Defective mismatch repair leads to the microsatellite instability (MSI) phenotype of colorectal cancer (CRC). We previously showed that the *MLH1*-93G>A promoter polymorphism is strongly associated with MSI tumours, suggesting a modifier role for this polymorphism in CRC. The *MLH1* promoter is bi-directional with the *EPM2AIP1* gene located on the antisense strand. In order to evaluate the functional effects of this polymorphism, we transfected a panel of CRC, endometrial cancer and non-tumourigenic cell lines with *MLH1* luciferase promoter constructs. We used constructs in reverse orientation to assess the effect of this polymorphism on *EPM2AIP1*. The luciferase activities were compared using a two-sided Student's t-test. Electrophoretic mobility shift assays (EMSAs) were used to evaluate whether differential protein binding was responsible for the differences in promoter activity. We observed a higher level of activity with the -93G allele in all the cell lines observed; including the CRC cell line, HCT116 (P=0.002), the endometrial cancer cell line, HEC-1-A (P<0.001) and the normal colonic cell line, CCD-841-CoTr (P=0.002). This polymorphism also affected *EPM2AIP1* transcription with the -93A allele demonstrating higher promoter activity in the HCT116 (P=0.007) and HEC-1-A (P=0.004) cells. The EMSA results suggest that this polymorphism alters the affinity of nuclear factors that bind to this region. Our findings indicate that the -93G>A polymorphism modifies the efficiency of *MLH1/EPM2AIP1* transcription.

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Introduction

With over a million cases diagnosed each year, colorectal cancer (CRC) is a significant cause of morbidity and mortality worldwide. CRCs can be generally subdivided into two clinically relevant groups depending on their mismatch repair (MMR) status, MMR-proficient and MMR-deficient CRCs. The DNA MMR system guards the integrity of the genome and contributes to the overall fidelity of DNA replication by targeting mispaired bases and insertion-deletion loops that occur through replication errors, during homologous recombination, and as a result of DNA damage (1). Cancers deficient in MMR exhibit genome-wide instability at microsatellite sequences in comparison to matched normal tissue DNA. When ≥30% of microsatellite markers tested show instability, the tumour is defined as high-frequency microsatellite instability (MSI-H).

About 15% of sporadic CRCs and >90% of Lynch syndrome (an inherited CRC syndrome) tumours display the MSI-H phenotype (2,3). Furthermore, the majority of MSI-H CRCs occur due to the epigenetic silencing of the *MLH1* promoter (4). MMR deficiency has also been shown to give rise to sporadic endometrial cancer with 28% of all endometrial tumours displaying a loss of MMR (5). Moreover, endometrial cancer is also the most common extra-colonic malignancy observed in the Lynch syndrome (6).

Evidence has shown *MLH1* to be a bi-directional promoter with a second gene, *EPM2AIP1*, located in a head-to-head orientation on the opposite strand 321 base pairs away from the *MLH1* transcription start site (7). The methylation of this promoter region results in the transcriptional silencing of both the *MLH1* and *EPM2AIP1* genes (7). Furthermore, MSI-H tumours show a significant reduction in *MLH1/EPM2AIP1* expression compared to normal colonic mucosa and microsatellite stable tumours, indicating that *EPM2AIP1* is concurrently turned off in the majority of sporadic tumours with *MLH1* hypermethylation (8). Although these findings potentially implicate *EPM2AIP1* in CRC, its functions to date remain unknown.

Our previous study examined the role of MMR single nucleotide polymorphisms (SNPs) in CRC and identified an association between the *MLH1*-93G>A promoter polymorphism and MSI-H CRCs in two separate Canadian

populations from the provinces of Ontario and Newfoundland (9). Since then, our findings have been confirmed by other studies, as this polymorphism has been shown to be associated with an increased risk of hyperplastic polyps and adenomas in smokers (10), as well as MSI-H CRCs (11). The *MLH1*-93G>A polymorphism has also been shown to be associated with colorectal tumours that display a loss of MLH1 protein expression (12). Furthermore, the *MLH1*-93G>A polymorphism has been shown to increase the risk of developing endometrial cancer (13). The correlation between the -93G>A SNP and cancer has not been limited to CRC or endometrial cancer, with previous studies reporting an association with an increased risk of ovarian (14) and squamous cell lung cancer (15). Taken together, these studies provide evidence that the -93G>A SNP is likely to mediate functional effects that promote carcinogenesis. However, there has been no systematic investigation of the functional contribution of this SNP, and the data that exist to date are conflicting (16-18). Furthermore, no study has evaluated the impact of this polymorphism in a panel of cell lines representative of CRCs and endometrial cancers, which represent the two most common types of cancer associated with MSI and MMR deficiency. This is particularly relevant as distinct functional contributions are likely to be observed in diverse cell types due to disparities in transcription factor expression and regulation (19).

In this study, we carried out a comprehensive investigation of the role of the -93G>A SNP in altering *MLH1* transcription in a panel of CRC, endometrial cancer and non-tumourigenic cell lines. In addition, we extended our analysis to ascertain whether this SNP affects the transcription of the *EPM2AIP1* gene, in an attempt to delineate the role of this gene in carcinogenesis.

Materials and methods

Promoter constructs. pGL3-basic plasmids of the *MLH1* promoter (ENST00000231790) were gifts from Dr Hiromichi Hemmi (Toho University School of Medicine, Japan). These plasmids, which contain either the G or A nucleotide at the -93 location, have been described previously (17).

In order to generate plasmids of the *MLH1* promoter encompassing the *MLH1*-93G>A polymorphism, DNA fragments corresponding to the -113 to +99 region (numbering is with reference to the *MLH1* translational start site) were PCR amplified from the constructs above. The PCR reaction contained 10X Taq buffer HiFi, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 mM primers, 14 ng template DNA, and 1 unit high-fidelity TaqDNA polymerase (Fermentas Life Sciences, Burlington, ON, Canada). The PCR conditions were as follows: Initial denaturation (95˚C for 10 min), denaturation (95˚C for 30 sec), annealing (57˚C for 45 sec), extension (72˚C for 1 min), and a final extension (72˚C for 8 min). The denaturation-annealing-extension steps were repeated 35 times. The 5' to 3' sequences of the forward and reverse primers, respectively, were: AAAAAAAACTCGAGGGATGGCGTAAGCTA (*Xho*I site underlined), AAAAAAAAAAGCTTCTTTGATAGCAT TAGCTGGCCG (*Hin*dIII site underlined).

As the *EPM2AIP1* promoter has not been characterized, we generated promoter constructs that incorporate the shared region between the two genes. In order to generate plasmids with the *EPM2AIP1* promoter G or A allele, DNA fragments corresponding to the -513 to +122 region of the *MLH1*/*EPM2AIP1* promoter were amplified from lymphocyte DNA of CRC patients homozygous (for G or A) at the -93 locus, as genotyped previously (9). The PCR conditions were identical to those described for the *MLH1* promoter, except that 40 ng of template DNA and 2.5 mM $MgCl_2$, were used. The 5' to 3' sequences of the forward and reverse primers, respectively, were: CCTCGTCGACTTCCATCTTGCTTCTTTT and CCGTACCAGTTCTCAATCATCTCTTTGAT. A second, nested PCR, was performed using the PCR fragment generated above as the template. The PCR conditions were identical to the above reaction except that 20 ng of template DNA were used. The 5' to 3' sequences of the forward and reverse primers, respectively, were: AAAAAAAAAAGCTT CACAAGCCCGGTTCC (*Hin*dIII site underlined) and AAAAAAAACTCGAGAAACGTCTAGATGCTCAACGG (*Xho*I site underlined).

Following all PCR reactions, DNA was isolated by gel electrophoresis on 1% agarose gel and purified by the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON, Canada). *MLH1* and *EPM2AIP1* promoter constructs, and the pGL3-basic vector were digested with *Xho*I and *Hin*dIII restriction endonucleases (Fermentas). The restriction digestion reaction was carried out using 10X Buffer R with BSA, 12.5 units of each restriction enzyme, and a 3:1 insert to vector ratio. The reaction mix was incubated at 37˚C for 18 h. A second round of restriction enzymes was added at 8 h to ensure maximum digestion. Following the digestion, 5 μl of shrimp alkaline phosphatase were added in order to prevent the religation of the plasmid and the mixes were incubated at 37˚C for 1 h. Following enzyme inactivation at 80˚C for 20 min, DNA was purified using the QIAQuick PCR Purification Kit (Qiagen). The integrity of all the constructs was verified by DNA sequencing.

Cell culture. Tissue culture reagents and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Burlington, ON, Canada). HT29, HCT116 and HEC-1-A cells were cultured in McCoy's 5A medium. SW620 and SW480 cells were grown in Leibovitz's L-15 medium. Human embryonic kidney (HEK) 293T and CCD-841-CoTr cells were maintained in Dulbecco's Modified Eagle's Medium. SK-UT-1B was maintained in Eagle's minimum essential medium [American Type Culture Collection (ATCC), Manassas, VA]. All the cell culture media were supplemented with 10% FBS. All cell lines were incubated at 37˚C in a humidified atmosphere of 5% CO_2, except for CCD-841-CoTr, which were grown at 33˚C. All cell lines were obtained from the ATCC.

Luciferase reporter gene assays. All transfection experiments were carried out using Lipofectamine 2000 reagent (Invitrogen Life Technologies) according to manufacturer's instructions. Cells were plated in 24-well plates at a density of 100,000 cells per well 24 h prior to transfection. The promoter-less pGL3-basic (empty vector) was used as the negative control. Luciferase constructs (500 ng) containing either the pGL3-basic, the *MLH1* (-93G or A), or *EPM2AIP1* promoter

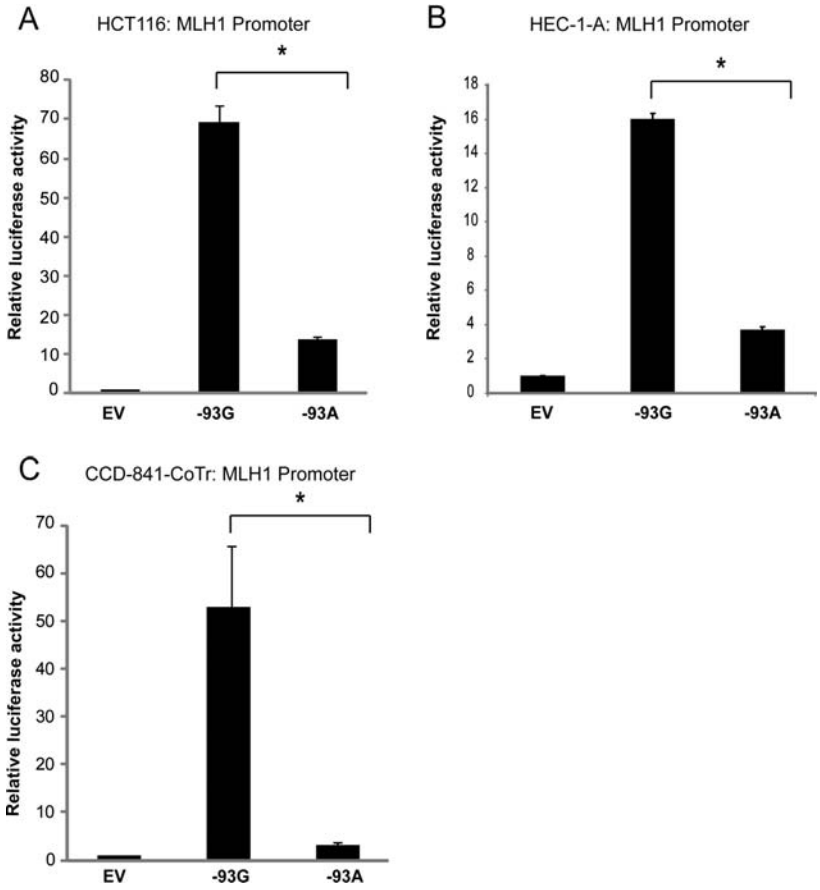

Figure 1. Effect of the *MLH1*-93G>A polymorphism on *MLH1* transcriptional activity. Effects of the -93G>A polymorphism were evaluated using luciferase constructs encompassing the *MLH1* promoter containing either the G or A allele at the -93 location. Promoter activities were assayed in the colon cancer cell line HCT116 (A), in the endometrial cancer cell line HEC-1-A (B), and in the normal colon cell line CCD-841-CoTr (C). The results shown for the -93 G or -93A allele were derived from firefly luciferase activity normalized to Renilla, and expressed relative to the EV plasmid. Each sample was assayed at least in triplicate, with three independent experiments performed. Statistically significant differences in promoter activity were assessed using the Student's t-test and are indicated by an asterisk. Error bars represent standard deviation from the mean. EV, empty vector (pGL3-basic) construct.

constructs (-93G or A), were transfected into the cells to determine luciferase reporter activity (17,20). Cells were additionally co-transfected with an internal control (5 ng of pRL-SV40, Promega, Madison, WI) for monitoring transfection efficiency and normalization. Luciferase and Renilla activities were measured 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega), which allows the firefly and Renilla luciferase activities to be quantified in the same sample. Luminescence was quantified using a Berthold 96-well microplate luminometer (Berthold, Wildbad, Germany). All reporter assays were performed at least in triplicate, with at least three independent experiments performed. Statistically significant differences in promoter activity were assessed using a two sided Student's t-test. Error bars represent standard deviation (SD) of the mean.

Electrophoretic mobility shift assays (EMSAs). The following double-stranded probes (SNP shown in bold within brackets) that were labeled with biotin on the 5' end were purchased from Integrated DNA Technologies (Coralville, IA): -93G sense, 5'-TAAGCTACAGCT[**G**]AAGGAAGAACGTG-3'; -93G antisense, 5'CACGTTCTTCCTT[**C**]AGCTGTAGC TTA-3'; -93A sense, 5'-TAAGCTACAGCT[**A**]AAG GAAGAACGTG-3'; -93A antisense, 5'- CACGTTCTTCCT T[**T**]AGCTGTAGCTTA-3'. The probes generated for the competition reaction were identical, except they did not contain a 5' biotin group.

Nuclear extracts of the cell lines were prepared using the NE-PER kit (Pierce, Rockford, IL) according to the manufacturer's instructions. EMSAs were carried out using the LightShift Chemiluminescent EMSA Kit (Thermo-Fisher, Rockford, IL) according to the manufacturer's instructions. We incubated 15 μg of nuclear proteins from the HCT116 cell line with 60 fmol biotin-labeled oligonucleotide for 25 min at room temperature in binding buffer (10 mM Tris, 50 Mm KCl, 1 mM dithiothreitol, pH 7.5), 50 ng/μl poly (dI-dC), 0.05% NP40, and 3.125% glycerol. Binding was competed by 200-, 400-, 800- and 2,000-fold excess unlabeled oligonucleotides. Binding complexes were resolved by electrophoresis using 5% TBE Criterion gels (Bio-Rad, Hercules, CA), transferred to Biodyne B pre-cut modified nylon membranes (Pierce), were UV cross-linked, and visualised using the Chemiluminescent Nucleic Acid Detection system (Pierce). The EMSAs were performed at least three times independently.

Results

Effects of the MLH1-93G>A SNP on MLH1 promoter activity. We examined the effects of the *MLH1*-93G>A promoter poly-

Table I. Summary of luciferase assay results.

Cell line	Relative luciferase activity -93G (SD)	Relative luciferase activity -93A (SD)	Fold change G/A	P-value
MLH1 promoter constructs				
HCT116	69.38 (4.32)	13.81 (0.59)	5.02	0.002
SW620	32.08 (3.02)	11.3 (1.02)	2.84	0.003
HT29	9.84 (0.67)	2.45 (0.44)	4.02	0.001
SW480	42.00 (2.21)	4.28 (0.13)	9.81	0.001
HEC-1-A	15.85 (0.34)	3.69 (0.16)	4.30	<0.001
SK-UT-1B	83.52 (2.45)	8.8 (1.56)	9.49	0.001
CCD-841-CoTr	52.85 (12.76)	2.98 (0.90)	17.73	0.02
HEK293T	7.13 (1.31)	2.15 (0.34)	3.32	0.01
EPM2AIP1 promoter constructs				
HCT116	139.73 (7.39)	222.73 (7.05)	0.63	0.007
SW620	61.68 (0.86)	76.04 (9.18)	0.81	0.02
HT29	16.94 (2.40)	26.13 (6.02)	0.65	0.05
SW480	39.51 (1.92)	50.70 (7.82)	0.78	0.09
HEC-1-A	26.94 (1.14)	34.77 (1.38)	0.77	0.004
SK-UT-1B	255.15 (11.83)	161.52 (37.19)	1.58	0.024
CCD-841-CoTr	22.19 (1.11)	24.71 (0.86)	0.90	0.15
HEK293T	13.25 (0.95)	16.23 (0.82)	0.82	0.09

The values for the -93 G or -93 A alleles were calculated relative to the empty pGL3-basic vector and P-values were calculated using a two-sided Student's t-test. SD, standard deviation from the mean.

morphism on *MLH1* promoter activity using the *MLH1* promoter constructs (-113 to +99). The activity of the -93G allele in the HCT116 cell line was ~5 fold higher than the -93A allele (P=0.0025, Fig. 1A). Similar results, where significantly higher promoter activity was associated with the -93G allele compared to the -93A allele, were also observed in the SW620 (P=0.003), SW480 (P=0.001) and HT29 (P=0.001) CRC cell lines. In the human endometrial cancer cell lines, HEC-1-A (Fig. 1B) and SK-UT-1B, the -93G allele showed a 4.3- (P=0.0005) and 9.5-fold (P=0.001) higher activity compared to the -93A allele. In the normal colonic CCD-841-CoTr (Fig. 1C) and human embryonic kidney HEK293T cell lines, the -93G allele showed a 17.7- (P=0.02) and 3.3-fold (P=0.01) increase in promoter activity, respectively. Promoter activities of all constructs are summarized in Table I.

Effects of the MLH1-93G>A SNP on EPM2AIP1 promoter activity. Due to the bi-directional nature of the *MLH1/EPM2AIP1* promoter, we evaluated the effects of this polymorphism using the *EPM2AIP1* promoter constructs. The -93G allele showed a statistically significant decrease in promoter activity compared to the -93A allele (Table I) in the HCT116 (0.6-fold; P=0.007), SW620 (0.8-fold; P=0.02) and HT29 (0.6-fold; P=0.05) CRC cell lines. A similar trend was observed in the SW480 cells, although statistical significance was not achieved. The results for HCT116 are depicted in Fig. 2A.

In the endometrial cancer cell line, HEC-1-A, the -93G allele displayed lower activity than the -93A allele (~0.8-fold; P=0.004), similar to the CRC cell lines. However, in the SK-UT-1B cell line, this trend was reversed, with the -93G allele showing statistically significantly higher activity (~1.6-fold; P=0.024). The results for HEC-1-A are depicted in Fig. 2B.

In the non-tumourigenic cell lines, HEK 293T and CCD-841-CoTr, the -93G allele also demonstrated lower levels of activity. However, these differences were not statistically significant. The results for CCD-841-CoTr are depicted in Fig. 2C.

Effect of the MLH1-93G>A promoter SNP on transcription factor binding. We carried out EMSA to assess whether the differences observed in the luciferase reporter activity between the -93G and the -93A allele were due to differential binding of nuclear factors. We used increasing amounts of unlabelled probe as a competitor to verify the specificity of binding interactions. In the reactions carried out with the nuclear extract of the HCT116 cell line, multiple factors bound to the labeled probe (Fig. 3). In addition, these factors exhibit different affinities and competitive binding, as the addition of the unlabelled competitor probe diminished certain interactions while strengthening others. When the binding patterns with the -93G probe were compared to those of the -93A probe, we observed that one of the factors (arrowhead, Fig. 3) had a higher affinity for the -93G probe.

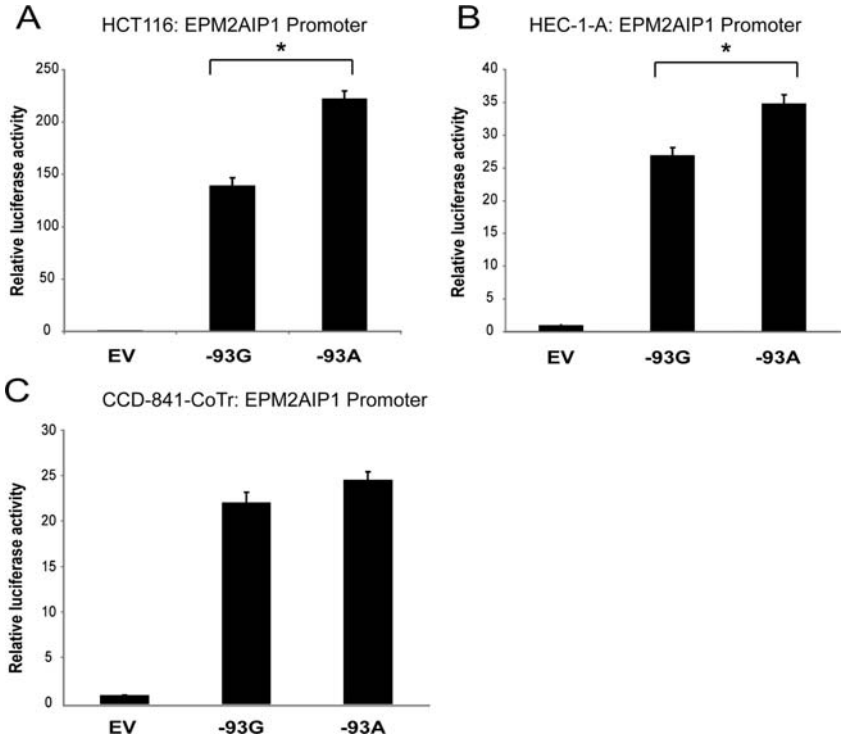

Figure 2. Effect of the *MLH1*-93G>A polymorphism on *EPM2AIP1* transcriptional activity. Constructs that span the shared bi-directional promoter, which contain either the A or G nucleotide at the -93 position (relative to *MLH1*) were transfected into the colon cancer cell line, HCT116 (A), the endometrial cancer cell line, HEC-1-A (B), and the normal colon cell line, CCD-841-CoTr (C). Results shown for the -93 G or A allele were derived from firefly luciferase activity normalized to Renilla, and expressed relative to the EV plasmid. Each sample was assayed at least in triplicate, with three independent experiments performed. Statistically significant differences in promoter activity were assessed using the Student's t-test, and are indicated by an asterisk. Error bars represent standard deviation from the mean. EV, empty vector (pGL3-basic).

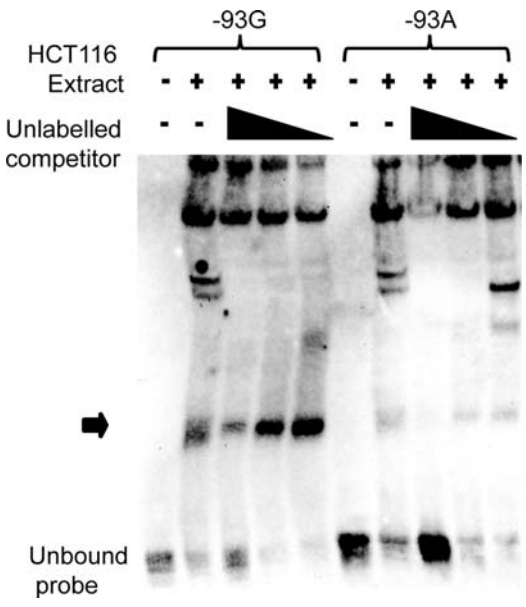

Figure 3. Role of the *MLH1*-93 >A polymorphism in affecting the binding of nuclear factors. An unlabelled competitor was added in decreasing concentrations (2000-, 800- and 400-fold, respectively) to verify the specificity and the affinity of the interactions. (+), reactions carried out in the presence of the HCT116 nuclear lysate; (-), reactions carried out without the nuclear extract or without the competitor. The arrowhead highlights factor(s) that show differential binding between the -93A and G alleles. At least three independent EMSAs were carried out to ascertain effects on binding and a representative experiment is depicted.

Furthermore, the highest concentration of competitor was unable to compete for binding to this factor for the -93G allele, while this was not so with the -93A allele. Similar results were observed when EMSA experiments were carried out with nuclear extracts from the CCD-841-CoTr cell line (data not shown).

Discussion

In this study, we show that the -93G>A promoter polymorphism affects the transcription of *MLH1* in cell lines derived from cancers of the colorectum, endometrium, as well as non-tumourigenic tissues. We consistently observed increased *MLH1* promoter activity for the -93G allele in all cell lines examined. Similar findings were observed in a recent study that investigated the effect of -93G>A on *MLH1* promoter activity. However, these studies were carried out in the human placental choriocarcinoma cell line JEG3 (18).

In addition to its role in *MLH1* transcription, analysis of the *EPM2AIP1* promoter constructs indicates that the -93G>A promoter polymorphism also alters the transcription of the *EPM2*AIP1 gene. Only the cancer cell lines examined demonstrated differences in the level of *EPM2AIP1* transcribed by the -93G and -93A alleles. With the exception of the SK-UT-1B cell line, the trend we saw was opposite to that of *MLH1*, with the -93A allele demonstrating higher activity. This result in the SK-UT-1B cell line could have arisen due to the cell type-specific expression of transcription

factors. These results indicate that while the overall effect of this SNP on the transcription of *EPM2AIP1* can differ between individuals and/or cell types, the presence of this polymorphism alters the level of *EPM2AIP1* transcribed. The -93G>A polymorphism is located in a region that has previously been shown to be crucial for *EPM2AIP1* transcription (7). Nonetheless, the functions of the *EPM2AIP1* gene are currently unknown (21). EPM2AIP1 has been found to interact with laforin (EPM2A), which has been implicated in the Wnt signalling pathway as well as in rapid onset tumourigenesis in mice (22). Nevertheless, it is unclear whether EPM2AIP1 plays a role in laforin-mediated tumourigenesis. Our results offer evidence for the first time that genetic alterations in the promoter region of *MLH1*, hitherto considered only for their potential effect on *MLH1* transcription, can potentially regulate the transcription of *EPM2AIP1* as well.

Given the observed effect of the -93G>A SNP on the transcription of both the *MLH1* and *EPM2AIP1* genes, we assessed how this SNP could influence transcription factor binding. Our EMSA results indicated that the region surrounding the -93G>A SNP was bound by multiple nuclear factors and that the -93G>A SNP altered the affinity and binding pattern of these factors in both the CRC and non-tumourigenic cell lines. These results corroborate the findings of our promoter activity assays. However, we could not determine whether the factors that bind to the region surrounding the -93G>A SNP are activators or repressors. Neither were we able to specifically delineate whether these factors regulate *MLH1*, *EPM2AIP1*, or both genes. Several studies have analyzed the promoter region of *MLH1* for potential transcription factor binding sites. However, the identity of the nuclear factors that bind to this region has not yet been determined. Based on the analysis using the GENETYX-SV software, one study showed that the -93G>A SNP occurs in putative transcription factor binding sites (16). However, a subsequent study using TFSEARCH and DNASIS software did not corroborate this finding (20). While knowing the identity of the factors would help shed further light on the mechanism by which this promoter polymorphism mediates its effects on gene transcription, identifying these factor(s) would not necessarily help interpret the risk associated with this SNP. The results from our EMSAs provide further evidence that the -93G>A SNP alters the transcription of the two genes, *MLH1* and *EPM2AIP1*.

A previous study found this SNP to be associated with *MLH1* methylation in endometrial and colorectal tumours (23,24), which raises the possibility that this polymorphism affects the binding of methylation machinery and therefore results in gene silencing. Thus, in addition to modifying the binding of transcription factors, this polymorphism may alter promoter methylation in a context-dependent manner. Furthermore, it is likely that these effects may not be independent of each other and could act in concert to regulate *MLH1*/*EPM2AIP1* transcription.

In conclusion, we demonstrate here that *MLH1*-93G>A is a functional polymorphism and that the -93 A allele results in decreased *MLH* transcription in a panel of CRC, endometrial cancer and non-tumourigenic cell lines. This builds on our previous finding that the -93A allele is associated MSI-H CRCs, and in combination with previously published genetic epidemiology studies, supports its role in modifying the risk of cancer development. Our results are particularly relevant as the cell lines we used are derived from CRCs and endometrial cancers, which represent the two most common types of cancer associated with MSI and MMR deficiency. Given that transcription factors are expressed in a cell type-specific manner, the potential impact of this polymorphism could vary between diverse cell types. This is also the first study to evaluate the effects of this SNP on the transcription of *EPM2AIP1*, demonstrating that it simultaneously affects both the regulation of *MLH1* and its antisense gene. Given that the functions of EPM2AIP1 are currently not known, this highlights a potential role for *EPM2AIP1* in carcinogenesis. The functional characterization of low risk alleles identified in population-based studies will enhance our understanding of cancer initiation and progression and could serve as potential tools for screening and prognosis.

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