

SNP rs16906252C>T Is an Expression and Methylation Quantitative Trait Locus Associated with an Increased Risk of Developing *MGMT*-Methylated Colorectal Cancer

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Abstract

Purpose: Methylation of the *MGMT* promoter is the major cause of *O*⁶-methylguanine methyltransferase deficiency in cancer and has been associated with the T variant of the promoter enhancer SNP rs16906252C>T. We sought evidence for an association between the rs16906252C>T genotype and increased risk of developing a subtype of colorectal cancer featuring *MGMT* methylation, mediated by genotype-dependent epigenetic silencing within normal tissues.

Experimental Design: By applying a molecular pathologic epidemiology case-control study design, associations between rs16906252C>T and risk for colorectal cancer overall, and colorectal cancer stratified by *MGMT* methylation status, were estimated using multinomial logistic regression in two independent retrospective series of colorectal cancer cases and controls. The test sample comprised 1,054 colorectal cancer cases and 451 controls from Sydney, Australia. The validation sample comprised 612 colorectal cancer cases and 245 controls from the Australasian Colon Cancer Family Registry (ACCFR).

To determine whether rs16906252C>T was linked to a constitutively altered epigenetic state, quantitative allelic expression and methylation analyses were performed in normal tissues.

Results: An association between rs16906252C>T and increased risk of developing *MGMT*-methylated colorectal cancer in the Sydney sample was observed [OR, 3.3; 95% confidence interval (CI), 2.0–5.3; *P* < 0.0001], which was replicated in the ACCFR sample (OR, 4.0; 95% CI, 2.4–6.8; *P* < 0.0001). The T allele demonstrated about 2.5-fold reduced transcription in normal colorectal mucosa from cases and controls and was selectively methylated in a minority of normal cells, indicating that rs16906252C>T represents an expression and methylation quantitative trait locus.

Conclusions: We provide evidence that rs16906252C>T is associated with elevated risk for *MGMT*-methylated colorectal cancer, likely mediated by constitutive epigenetic repression of the T allele. *Clin Cancer Res*; 22(24); 6266–77. ©2016 AACR.

Introduction

The DNA repair protein *O*⁶-methylguanine DNA methyltransferase (*MGMT*) plays a crucial role in protecting cells from the carcinogenic effects of alkylating agents by removing mutagenic alkyl adducts from DNA. *MGMT* molecules are degraded during

the repair process, so need constant replenishment via transcription. The most frequent mechanism of transcriptional inactivation of *MGMT* in cancer is aberrant methylation of its CpG island promoter (1, 2). *MGMT* methylation or reduced *MGMT* mRNA levels in tumors are predictors of chemosensitivity to alkylating

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

We present evidence for an association between the rs16906252C>T SNP genotype and increased risk of developing colorectal carcinoma exhibiting *MGMT* methylation, coupled with reduced risk of developing colorectal cancer unmethylated at *MGMT*. This has translational implications for cancer risk stratification. Carriers of the rs16906252 T variant are more susceptible to developing a *MGMT*-methylated colorectal cancer, with ORs of magnitude 3.3 to 4.0, presumably upon exposure to alkylating agents. We provide evidence that this increased risk association for *MGMT*-methylated colorectal cancer is functionally mediated by epigenetic alteration of alleles bearing the T variant, including constitutively reduced transcriptional activity and somatic methylation, within normal tissues such as blood and colorectal mucosa. The T variant has only been identified among Europeans and South Asians, hence these findings are most relevant to Caucasians.

chemotherapy in patients with cancer (3–6). Therefore, it would be of translational interest to determine the factors, including genetic risk variants, which affect the expression and/or the methylation status of *MGMT*, as these could also influence the outcomes of treatment with alkylating chemotherapy (7, 8).

Colorectal cancer is heterogeneous and may be subdivided into 4 major molecular subtypes, based on gene expression and methylation profiles, and enrichment for particular genetic alterations, consistent with 4 distinct neoplastic pathways (9, 10). One subtype exhibits the CpG island methylator phenotype (CIMP)-high involving methylation of numerous CpG islands genome-wide, BRAF V600E mutation, and high rates of microsatellite instability (11, 12). Another subtype features CIMP-low, in which a subset of CIMP-high loci is methylated, and enrichment for *KRAS* mutations (12, 13). The remaining subtypes are CIMP-negative with chromosomal instability (9, 10). *MGMT* methylation occurs in around 25% to 40% of colorectal cancer (1, 2) and has been associated with *KRAS* mutations (2, 14–20), particularly G>A transitions (14, 15, 17, 18). However, no consistent correlation between *MGMT* methylation and CIMP status has been found (2, 9, 12, 20). *MGMT* methylation thus cross-sections the major molecular subtypes of colorectal cancer (9, 12), and the mechanisms underlying it remain undefined.

We and others have found a close association between carriage of the T variant of the germline SNP rs16906252C>T within the *MGMT* promoter enhancer region and the presence of *MGMT* methylation in colorectal cancer (2, 21), lung adenocarcinoma (22), pleural mesothelioma (23), glioblastoma (8, 24), and large cell B lymphoma (25). We and others have also reported low levels of *MGMT* methylation within normal tissues of cancer cases and healthy controls in association with the T variant (2, 26). The initial finding of low-level *MGMT* methylation within the macroscopically normal colorectal mucosa (NCM) of colorectal cancer cases led to speculation this may serve as a field defect preceding and predisposing to colorectal cancer (16), although the association with rs16906252C>T was unrealized at that time. Consistent with the concept of field cancerization (27), we showed that low-level *MGMT* methylation within the NCM of

colorectal cancer cases was more commonly detected in those whose tumor was correspondingly methylated (2). Furthermore, we demonstrated that low-level *MGMT* methylation within the NCM of both colorectal cancer cases and controls was associated with the rs16906252C>T genotype (2). Low-level *MGMT* methylation has also been detected in the peripheral blood lymphocytes (PBL) of healthy controls, wherein the methylation was associated with rs16906252C>T and linked specifically to alleles bearing the T variant (26).

The functional mechanism underlying the association between the rs16906252C>T genotype and *MGMT* methylation remains unknown. Of potential functional relevance, rs16906252C>T is a synonymous SNP located in exon 1 within a *cis*-acting enhancer element that is essential for *MGMT* upregulation (28, 29). Reduced transcription has been identified as one mechanism underlying permanent epigenetic inactivation via the accumulation of repressive histone modifications, and ultimately, promoter methylation (30). Promoter reporter assays performed in lung adenocarcinoma and glioblastoma cell lines have shown reduced activity from the T, compared with the wild-type C, allele at rs16906252C>T (8, 22). The effect of rs16906252C>T on transcriptional activity has not been systematically investigated *ex vivo*, although reduced levels of transcripts derived from the T allele was reported in bronchial epithelial cells from 2 heterozygous cancer-free individuals (22).

We hypothesized that the rs16906252C>T variant is associated with an elevated risk of developing an *MGMT*-methylated subtype of CRC, functionally mediated via constitutively reduced transcription from the T allele and its susceptibility to somatic methylation. Using a molecular pathology epidemiology retrospective case-control study design, we tested our hypotheses in a test sample of 1,054 colorectal cancer cases, which we stratified by *MGMT* methylation status of the tumors, and 451 controls from Sydney, Australia, and replicated key observations in an independent sample of 612 cases and 245 controls from the Australasian Colon Cancer Family Registry (ACCFR). We also sought to elucidate the mechanism underlying the association between rs16906252C>T and *MGMT* methylation in colorectal cancer by investigating constitutive allelic methylation and expression profiles within normal tissues from subsets of cases and controls.

Materials and Methods

Subjects and specimens

An overview of the study design and inclusion of subjects and specimens is provided in Fig. 1. Specimens of primary colorectal cancer and subject-matched NCM and/or PBL were drawn from 2 independent series of colorectal cancer cases from Australia. The "Sydney CRC series" comprised a sample of 1,054 colorectal cancer cases drawn from 1,178 patients who had undergone surgical resection of a primary colorectal cancer at St Vincent's Hospital Sydney, between 1993 and 2008 (Supplementary Table S1), including 1,039 patients in whom we previously reported a close association between the presence of *MGMT* methylation in colorectal cancer and rs16906252C>T (2). Cases identified with germline mutations within the mismatch repair genes, *APC* and *MUTYH* were omitted. The "ACCFR CRC series" comprised 612 cases diagnosed with incident colorectal cancer between 1997 and 2008, for whom both blood and tumor were available, recruited

via the Victorian Cancer Registry, a population registry for the state of Victoria (Supplementary Table S2; ref. 31). For patients who developed 2 or more primary colorectal cancer, one cancer was selected at random for inclusion in this study. PBL was obtained from 2 groups of control subjects without a personal history of cancer recruited from the same catchment area as the respective colorectal cancer series. Controls for the "Sydney CRC series" comprised 451 blood donors recruited at St Vincent's Hospital Sydney via the Australian Red Cross Blood Service (ARCBS), whose rs16906252C>T genotypes we have previously reported (8). Controls for the "ACCFR CRC series" comprised 245 individuals randomly recruited from the general population via Victorian Electoral Rolls and were additionally age- and sex-frequency matched to the cases (31). All subjects were derived from predominantly Caucasian populations.

For *ex vivo* functional analyses of the rs16906252C>T SNP, subsets of cases from the "Sydney CRC series" and "ARCBS controls" were selected on the basis of genotype. NCM from 3 cancer-free subjects who underwent colonic resection at St Vincent's Hospital Sydney for indications other than colorectal cancer and who we previously identified as rs16906252C>T heterozygotes (2) were included as controls.

This study was approved by Human Research Ethics Committees of The University of New South Wales St Vincent's Campus (H02/022, H07/002), The University of Melbourne (#020702), and ARCBS (2003#08). All subjects provided signed informed consent prior to participation.

Genotyping

Genotyping of rs16906252C>T was performed on DNA extracted from PBL or NCM, either by PCR amplification followed by *HhaI* restriction fragment length polymorphism (RFLP) analysis, as previously described (2, 24) or by using Sequenom's iPLEX Gold. The genotyping assay was designed using MassARRAY Assay Design 3.0 software (Sequenom, Inc.) and performed according to the manufacturer's instructions. Extension product sizes were determined using Sequenom's Compact MALDI-TOF mass spectrometer. The resulting mass spectra were converted to genotype data using SpectroTYPER-RT software.

Methylation analyses

DNA was converted with sodium bisulphite using the EZ DNA Methylation Gold kit (Zymo Research). For tumors, *MGMT* methylation status was determined by COBRA in the Sydney CRC series and by MethyLight in the ACCFR CRC series, as previously described (2, 11, 19). Tumors with methylation levels of $\geq 5\%$ (COBRA) or a percentage of methylated reference (PMR) value > 4.0 (MethyLight) were considered "methylated," as this threshold correlates most closely with protein loss (32).

As previously described (2), a sensitive real-time methylation-specific PCR (qMSP) assay was used to measure *MGMT* methylation in matched PBL, NCM, and colorectal cancer from a subset of colorectal cancer cases and PBL from a subset of controls (Fig. 2A). Tumors were categorized as "methylated" when the PMR > 4.0 . PBL and NCM were categorized as "methylation-low" when PMR ≥ 0.01 (the analytical sensitivity threshold for this assay).

To determine whether methylation-low detected by q-MSP within normal tissues of rs16906252C>T heterozygotes was linked to a specific rs16906252 allele, an allelic-MSP assay encompassing rs16906252 was performed using primers 5'-

GGTTGTATCGTTTCGAGGGAGAGTT-3' and 5'-CCGCGCCC-CGAATATACTAAA-3' (Fig. 2A). Amplicons were cloned using the pGEMTeasy PCR cloning system (Promega), and the inserts from 12 to 24 colonies sequenced.

Promoter reporter assays

Luciferase promoter reporter constructs containing the *MGMT* promoter sequence, with either the wild-type C (HAP1) or the variant T (HAP4) allele at rs16906252 inserted into the pGL2-basic vector (22), were transiently transfected into human embryonal kidney (HEK293) and colorectal cancer (HCT116) cell lines. The levels of luciferase reporter activity were measured by luminescence (Supplementary Methods).

Allelic expression analyses

To measure the relative levels of transcripts derived from the C and T alleles at rs16906252C>T, allele quantification (AQ) assays were performed in cDNAs from NCM of selected colorectal cancer cases and controls either directly by *HhaI* AQ-RFLP analysis of rs16906252 at c.66 or indirectly at the linked rs1803965C>T SNP at c.252 within exon 3 by AQ-pyrosequencing, and subsequently ascertaining the rs16906252;rs1803965 haplotypes by clonal sequencing of cDNA fragments encompassing both SNPs (Supplementary Methods and Supplementary Fig. S1). SNPs are located with reference to GenBank accession NM_002412.3. To ascertain haplotypes between rs16906252C>T of functional interest and the downstream rs1803965C>T SNP assayed, RT-PCR was performed across exons 1 and 3 encompassing both SNPs in cDNAs from double heterozygotes using primers 5'-CCGGATATGCTGGGACAG-3' and 5'-CTGGTGAAC-GACTCTGTCTG-3'. Products were cloned in pGEMTeasy to isolate individual transcripts, and the inserts sequenced using vector primers.

For both AQ assays, the fold difference in the levels of rs16906252C:T transcripts were normalized against the levels of the respective alleles in genomic DNA and calculated as an allelic expression ratio (AER) by: $(C_{cDNA}/T_{cDNA})/(C_{DNA}/T_{DNA})$ (33). Following haplotyping of double heterozygotes, the AERs obtained by AQ-pyrosequencing at rs1803965C>T in exon 3 were inverted, where necessary, to reflect the C:T AER at rs16906252C>T upstream.

Statistical analyses

MGMT methylation status was analyzed as a categorical variable except where stated. *P* values for unadjusted ORs were estimated using the χ^2 test. Multivariate unconditional logistic regression was used to determine independent factors among significant, unadjusted covariates. Where more than one factor was associated with presence of *MGMT* methylation, logistic regression was used to obtain adjusted ORs. A multinomial logistic regression model was used to compare the subsets of colorectal cancer cases stratified by *MGMT* methylation status, with healthy controls as the referent group. Nonnormally distributed, continuous PMR and AER values were compared between groups using a Mann-Whitney *U* test or between subject-matched samples using a related-samples Wilcoxon signed rank test. Luciferase expression values were compared using the Student *t* test. A correlation between AER and PMR values was estimated using Spearman ρ . All statistical tests were 2-sided.

Online tools

The minor allele frequency (MAF) of rs16906252C>T was obtained from data accessed from dbSNP via the NCBI website <http://www.ncbi.nlm.nih.gov/projects/SNP/> and the 1000 Genomes Project at <http://browser.1000genomes.org/index.html>.

Results**Confirmation of the association between *MGMT* methylation in colorectal cancer and rs16906252C>T**

In our test series of 1054 Sydney CRC cases, 292 tumors (27.7%) were positive for *MGMT* methylation. We found a clear association between the presence of *MGMT* methylation in colorectal cancer and carriage of the T variant at rs16906252C>T in univariate analysis [OR, 6.23; 95% confidence interval (CI), 4.3–8.9; $P < 0.0001$; Table 1], with strong evidence for an independent association in multivariate analyses (OR, 8.0; 95% CI, 5.3–12.1; $P < 0.0001$; Supplementary Table S1), recapitulating our previous findings among 1,039 of these cases (2). In our validation series of 612 colorectal cancer cases from the ACCFR (Supplementary Table S2), 129 tumors (21.1%) were *MGMT*-methylated, and a clear association between *MGMT* methylation and the rs16906252C>T genotype was found (OR, 7.12; 95% CI, 4.3–11.7; $P < 0.001$; Table 1). In both series, these associations remained significant when adjusted for age and gender (Table 1). Our consistent findings in 2 independent colorectal cancer series provide confirmatory evidence for a close association between rs16906252C>T and *MGMT* methylation in colorectal cancer. The rs16906252C>T genotype had low sensitivity but high specificity for an *MGMT*-methylated tumor (Supplementary Table S3).

The rs16906252C>T genotype is associated with increased risk of developing *MGMT*-methylated colorectal cancer

To determine whether the rs16906252C>T genotype was associated with an increased risk of colorectal cancer development, we firstly compared the distribution of genotypes between the cases and controls in the test sample (Fig. 1A). The MAF of rs16906252C>T in the Sydney CRC series was 7.5%, and 14.9% of patients carried the T variant (CT/TT genotypes combined). In the respective ARCBS controls, the MAF was 6.1%, and 12% carried the T variant (8). The SNP genotype frequencies were in Hardy–Weinberg equilibrium in both the colorectal cancer ($P = 0.21$) and the control ($P = 0.86$) groups. Carriage of the T variant was not associated with risk for colorectal cancer overall (Table 3). Next, in a molecular pathologic epidemiology study design, we determined whether the rs16906252C>T genotype was associated with risk of developing colorectal cancer specifically exhibiting

MGMT methylation in a subtype-specific manner. We stratified the colorectal cancer cases by the *MGMT* methylation status of their tumor into *MGMT*-methylated or *MGMT*-unmethylated categories and compared the distribution of genotypes in these 2 categories with the control group. Carriage of the T variant was associated with an increased risk for *MGMT*-methylated colorectal cancer (OR, 3.3; 95% CI, 2.0–5.3; $P < 0.0001$), coupled with a reduced risk for an *MGMT*-unmethylated colorectal cancer (OR, 0.5; 95% CI, 0.3–0.9; $P = 0.01$; Table 2).

To confirm these observations, we repeated the analyses in the validation sample (Fig. 1A). The MAF in the ACCFR CRC series was 7.0%, and 13.6% carried the T variant. The MAF in the matched control group was 6.3%, and 12.7% carried the T variant (Table 2). The SNP genotype frequencies were in Hardy–Weinberg equilibrium in both cases ($P = 0.99$) and controls ($P = 0.29$). The rs16906252C>T genotype was not associated with risk for colorectal cancer overall; however, rs16906252C>T was associated with an elevated risk for *MGMT*-methylated colorectal cancer (OR, 4.0; 95% CI, 2.4–6.8; $P < 0.0001$) and a reduced risk for *MGMT*-unmethylated colorectal cancer (OR, 0.5; 95% CI, 0.3–0.9; $P = 0.02$; Table 2). Hence, the associations we observed between the rs16906252C>T genotype and elevated risk of developing *MGMT*-methylated colorectal cancer and reduced risk of *MGMT*-unmethylated colorectal cancer in the test sample were replicated with similar magnitude in the validation sample.

The rs16906252 T genotype underlies low-level methylation in normal tissues from colorectal cancer cases

We next sought to elucidate the mechanistic basis for the risk association identified between rs16906252C>T and development of an *MGMT*-methylated cancer. We determined whether (i) low-level *MGMT* methylation was detectable systemically or specifically within the NCM of colorectal cancer cases; (ii) this was associated with rs16906252C>T genotype; and (iii) methylation or genotype were predictive of the *MGMT* methylation status of the corresponding tumor. We used sensitive qMSP (Fig. 2A) to measure the *MGMT* methylation levels in matched PBL, NCM, and tumor tissues from a subset of cases from the Sydney CRC series. To maximize the number of cases with the rs16906252C>T genotype, all heterozygotes from this series were considered eligible for inclusion and those with availability of all 3 tissue types were included ($n = 30$; Fig. 1B). A similar number ($n = 32$) of C/C homozygotes were then selected from the series at random.

(A) Low levels of *MGMT* methylation were detected in either or both PBL and NCM of a proportion of cases. Figure 2B shows the actual PMR scores for the matched tissues from each case

Table 1. Association between rs16906252C>T genotype and presence of *MGMT* methylation in colorectal cancer from 2 independent series of colorectal cancer cases

	Cases n (%)	CC n (%)	CT/TT n (%)	OR (95% CI)	P	_a OR (95% CI)	P
Sydney CRC series							
<i>MGMT</i> methylation	1,054	897 (85.1)	157 (14.9)				
Unmethylated	762 (72.3)	704 (92.4)	58 (7.6)	1.0 (referent)			
Methylated	292 (27.7)	193 (66.1)	99 (33.9)	6.2 (4.3–8.9)	<0.0001	6.3 (4.4–9.1)	<0.0001
ACCFR CRC series							
<i>MGMT</i> methylation	612	529 (86.4)	83 (13.6)				
Unmethylated	483 (78.9)	447 (92.5)	36 (7.5)	1.0 (referent)			
Methylated	129 (21.1)	82 (63.6)	47 (36.4)	7.1 (4.3–11.7)	<0.0001	7.1 (4.3–11.8)	<0.0001

Abbreviation: _aOR, OR adjusted for age and gender.

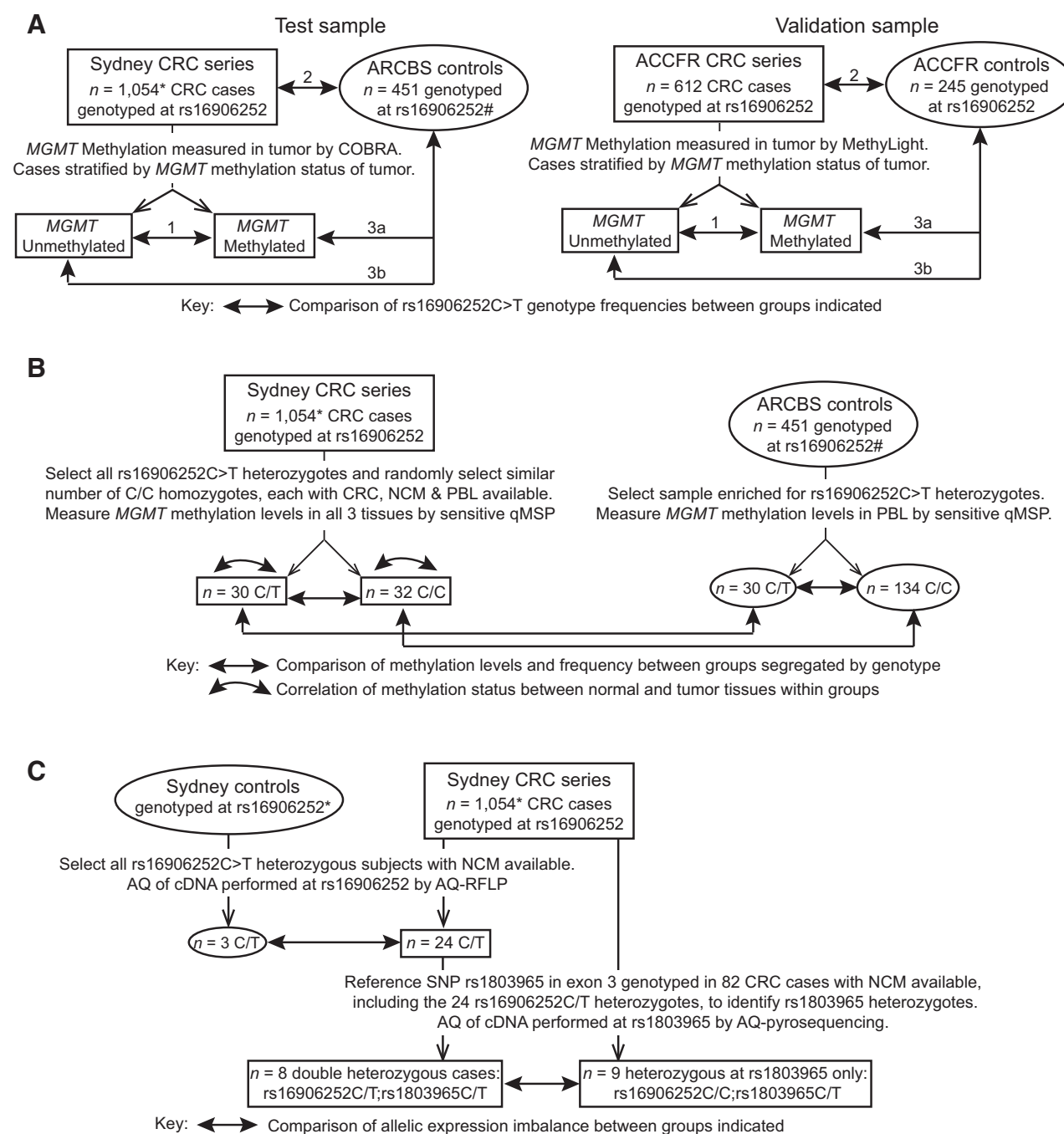


Figure 1.

Overall study design showing selection of cases and controls and usage of specimens. **A**, Molecular pathologic epidemiology study designed to estimate associations between rs16906252C>T SNP genotype and risk for MGMT methylation in colorectal cancer (CRC), as well as risk for CRC by MGMT methylation subtype. The test sample comprised 1,054 CRC cases who underwent surgery with curative intent at St Vincent's Hospital Sydney, designated the "Sydney CRC series," and a control group of geographically matched blood donors with no personal history of cancer who were recruited at St Vincent's Hospital Sydney by the ARCBS, designated "ARCBS controls." *, Of the 1,054 cases from the Sydney CRC series, 1,039 were previously included in our report of a close association between the rs16906252C>T genotype and the presence of MGMT methylation in the tumor (2). However, 50 cases included in both the prior and the present study presented with more than one synchronous or metachronous CRC and one tumor was selected at random for inclusion in each study. Hence, there is incomplete overlap in the tumors from these 50 cases between the 2 studies. #, We previously published the rs16906252C>T genotypes for the ARCBS controls in a study that assessed the association between rs16906252C>T genotype and risk of development of MGMT-methylated glioblastoma (8). The validation sample comprised 612 CRC cases from the ACCFR, designated the "ACCFR CRC series," and 245 controls without a personal history of cancer recruited from the Victorian electorate who were geographically and age and sex frequency matched to the cases, designated "ACCFR controls" (31). (Continued on the following page.)

Table 2. Statistical comparisons of the MGMT rs16906252C>T genotype between 2 independent series of colorectal cancer cases and their matched control groups

	n (%)	CC n (%)	CT/TT n (%)	aOR (95% CI)	P
Sydney (test sample)					
ARCBS controls	451	397 (88.0)	54 (12.0)	1.0 (referent)	
All CRC cases	1,054	897 (85.1)	157 (14.9)	1.1 (0.7-1.6)	0.73
MGMT-methylated CRC cases	292 (27.7)	193 (66.1)	99 (33.9)	3.3 (2.0-5.3)	<0.0001
MGMT-unmethylated CRC cases	762 (72.3)	704 (92.4)	58 (7.6)	0.5 (0.3-0.9)	0.01
ACCFR (validation sample)					
Controls	245	214 (87.4)	31 (12.7)	1.0 (referent)	
All CRC cases	612	529 (86.4)	83 (13.6)	1.1 (0.7-1.7)	0.75
MGMT-methylated CRC cases	129 (21.1)	82 (63.6)	47 (36.4)	4.0 (2.4-6.8)	<0.0001
MGMT-unmethylated CRC cases	483 (78.9)	447 (92.5)	36 (7.5)	0.5 (0.3-0.9)	0.02

NOTE: When the colorectal cancer cases were stratified by the MGMT methylation status in their primary carcinoma, carriage of the T allele was significantly associated with the presence of methylation in tumors (highlighted in bold).

Abbreviation: aOR, ORs have been adjusted by age group and gender using a multinomial logistic regression model.

segregated by rs16906252 genotype. (B) In categorical analyses, methylation-low (PMR \geq 0.01) was detected in the PBL of 9.4% of C/C and 73.3% of C/T cases. In NCM, methylation-low was detected in 15.6% of C/C and 93.3% of C/T cases (Table 3). A strong association between rs16906252C>T genotype and presence of methylation was identified for all 3 tissue types ($P < 0.0001$; Supplementary Table S4). (C) To determine whether the presence of methylation-low in PBL and/or NCM, or rs16906252C>T genotype, was predictive of MGMT methylation status in the corresponding tumor, we compared the strengths of their associations. Methylation-low in PBL or NCM and rs16906252C>T genotype showed moderate sensitivity and specificity for methylated tumors (Supplementary Table S5). Both the T genotype and the presence of methylation in PBL or NCM were strongly associated with tumor methylation (ORs, 11.9, 9.9, and 7.7, respectively; Table 3). However, the association for methylation-low in PBL and NCM, individually, declined significantly when adjusted for genotype (adjusted OR, 3.7, $P = 0.08$ and adjusted OR, 1.6, $P = 0.6$, respectively), whereas the risk for genotype adjusted by methylation-low status showed little change (PBL: adjusted OR, 5.8; NCM: adjusted OR, 8.3; Table 3). When compared with patients with

unmethylated normal tissues, those with methylation-low in both NCM and PBL showed a strong association with methylated tumors (OR, 13.9). This association also diminished significantly when adjusted for genotype (adjusted OR, 3.3), whereas the genotype association remained borderline statistically significant when adjusted by methylation status (adjusted OR, 6.4; Table 3).

On the basis of this subset of colorectal cancer cases, rs16906252C>T genotype appears to be the primary driver of MGMT methylation in colorectal cancer. Nevertheless, when actual PMR scores in normal tissues were analyzed as a continuous variable segregated by rs16906252C>T genotype, C/T cases had significantly higher levels of MGMT methylation compared with C/C cases in both PBL ($P < 0.0001$) and NCM ($P < 0.0001$) (Fig. 2B). Furthermore, within subjects, the levels of MGMT methylation were significantly higher in NCM compared with PBL among C/T cases ($P < 0.0001$), but not in C/C cases (Fig. 2B). The latter findings support the hypothesis that, in the setting of colorectal cancer, the T variant confers susceptibility to MGMT methylation within normal tissues, and this occurs at higher levels in NCM than in PBL.

(Continued.) Where genotyping had not already been performed, all cases and controls were genotyped at the rs16906252 SNP in germline DNA extracted from PBLs or paired NCM. For CRC cases, MGMT methylation status in the tumor was assayed by COBRA or MethyLight, and the cases were stratified as "methylated" or "unmethylated." Double arrow lines indicate groups between which the frequencies of carriage of the T variant at rs16906252 (C/T and T/T combined) were compared in both the test and validation samples to estimate associations between: (i) presence of MGMT methylation within tumors and rs16906252C>T among CRC cases only; (ii) rs16926252C>T and risk of CRC overall; (iii) rs16926252C>T and risk of developing a CRC that was (a) MGMT-methylated or (b) MGMT-unmethylated. **B** and **C**, *Ex vivo* functional analyses of the rs16906252C>T SNP. **B**, Detection of MGMT methylation within normal tissues and its association with rs16906252C>T. A sample of CRC cases with tumor, NCM, and PBL available was selected from the Sydney CRC series to determine whether MGMT methylation was detectable in either/both normal tissues and whether this and/or genotype was associated with the presence of MGMT methylation in the corresponding tumor (curved double arrows). To enrich for rs16906252C>T cases, all CRC cases heterozygous for the rs16906252C>T SNP were considered eligible for inclusion and 30 cases had all 3 tissue types available. A similar number of cases homozygous for the wild-type C allele at rs16906252 with all 3 tissue types available were selected at random to serve as a comparison group. MGMT methylation levels were measured using the semiquantitative and highly sensitive real-time qMSP technique. To determine whether low-level MGMT methylation detected in PBL was driven by cancer status or by genotype, a subset of ARCBS controls enriched for the rs16906252C>T genotype was tested by qMSP. The levels and frequency of methylation were compared between the cases and controls (straight double arrows). **C**, Quantitative allelic expression analyses in NCM to determine whether the variant T allele at rs16906252C>T was associated with constitutively reduced levels of transcription compared to the C allele. First, 3 cancer-free controls from St Vincent's Hospital, Sydney and 24 CRC cases from the Sydney CRC series who were all rs16906252C>T heterozygotes with fresh-frozen NCM tissue available were analyzed by AQ-RFLP at the rs16906252 SNP site (Supplementary Methods) in cDNAs from NCM to determine the relative levels of expression of the C and T alleles. The normalized C:T AERs were determined, which indicated an AEI with reduced expression from the T allele. The AERs were compared between the cases and controls (double arrow). To confirm the AEI observed by AQ-RFLP, an indirect method of measuring allelic expression levels was then performed by AQ pyrosequencing at a common, benign reference SNP, rs1803965C>T, located downstream in exon 3. To identify rs1803965C>T heterozygotes, 82 cases from the Sydney CRC series, including the 24 rs16906252C>T heterozygous cases analyzed by AQ-RFLP, were genotyped at rs1803965C>T. A subset of 8 of 24 of the rs16906252C>T heterozygous cases were double heterozygotes for the 2 SNPs. These 8 cases were additionally assessed for AEI by AQ-pyrosequencing at the exon 3 rs1803965C>T SNP. Nine cases heterozygous for the rs1803965 SNP assayed, but homozygous C at rs16906252 of functional interest, were included for comparison of the degree of AEI (double arrow).

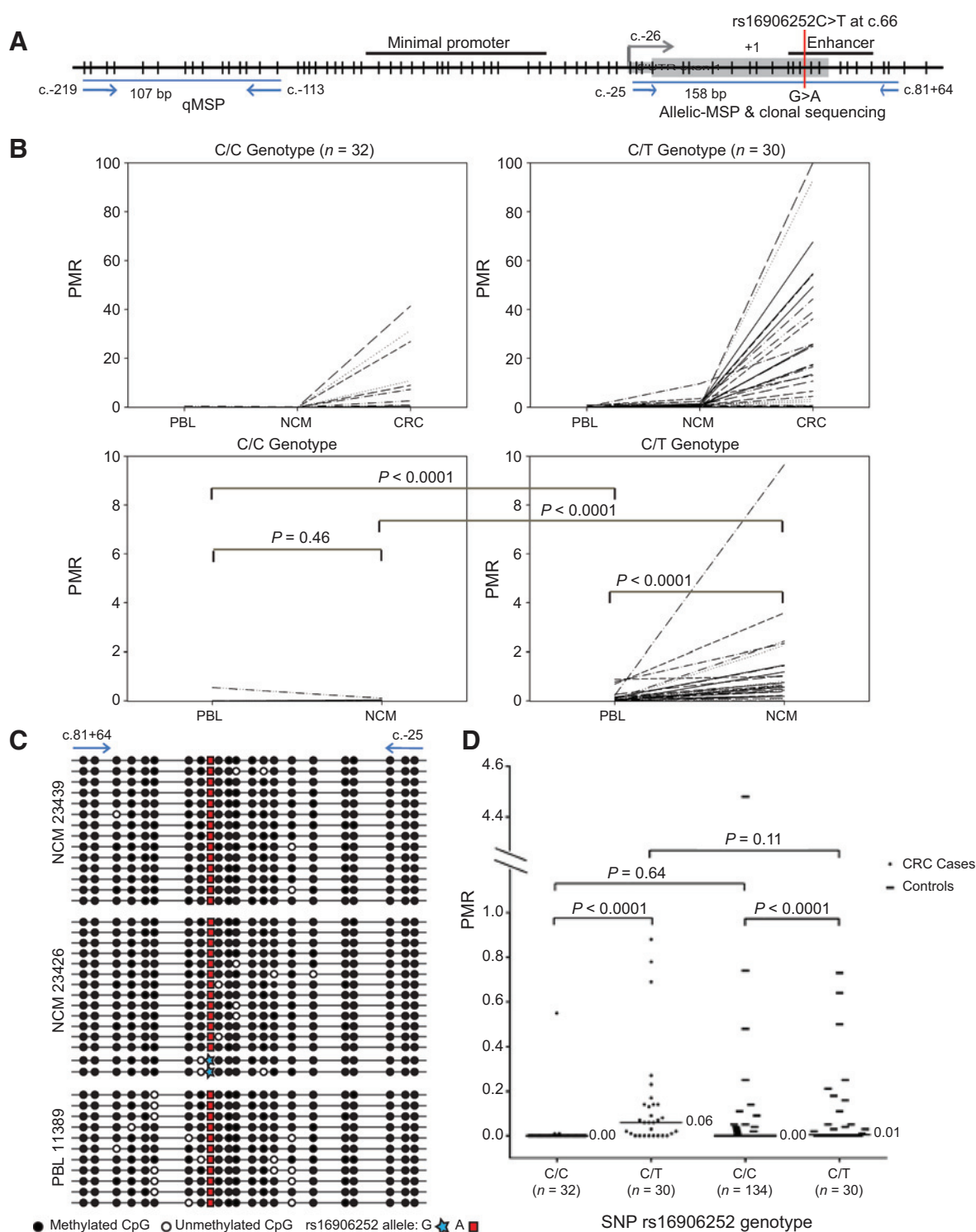


Figure 2. Methylation levels detected in subject matched normal and tumor tissue samples from colorectal cancer (CRC) cases by quantitative real-time MSP segregated by rs16906252 genotype. **A**, Map of the *MGMT* CpG island promoter and location of the rs16906252 SNP showing the positions of high-sensitivity MSP assays used to detect methylation of the *MGMT* promoter. The map is drawn with respect to the first ATG translation start site located at +1, according to *MGMT* consensus sequence (GenBank accession NM_002412.3). The 5' untranslated region of exon 1 is represented as a thin grey box, the coding region as a wide grey box, and the grey arrow indicates the transcription start site. CpG dinucleotides are shown as vertical black lines. Horizontal black lines indicate regulatory elements. Vertical red line indicates the location of the rs16906252C>T SNP, which is detected as a G>A change on the complementary strand. Blue lines show assay maps. (Continued on the following page.)

Table 3. Comparative associations between the rs16906252C>T genotype and the presence of low-level *MGMT* methylation in normal tissues with presence of *MGMT* methylation in primary colorectal cancer

	Unmethylated tumor (n = 34) n (%)	Methylated tumor (n = 28) n (%)	OR (95% CI)	P	^a OR (95% CI)	P
PBL						
PMR < 0.01	28 (82.3%)	9 (32.1%)	9.9 (3.0–32.3)	<0.001	3.7 (0.9–15.4) ^a	0.075
PMR ≥ 0.01	6 (17.7%)	19 (67.9%)				
NCM						
PMR < 0.01	23 (67.7%)	6 (21.4%)	7.7 (2.4–24.3)	<0.001	1.6 (0.3–9.7) ^a	0.600
PMR ≥ 0.01	11 (32.4%)	22 (78.6%)				
Methylation-low						
PBL and NCM	5 (14.7%)	19 (67.9%)	13.9 (3.7–53.0)	<0.001	3.3 (0.4–23.5) ^a	0.243
PBL only	1 (2.9%)	0 (0.0%)	NT	—	NT	—
NCM only	6 (17.7%)	3 (10.7%)	1.8 (0.4–9.6)	0.473	0.5 (0.06–5.0) ^a	0.596
Neither (referent)	22 (64.7%)	6 (24.4%)	1.00		1.00	
Genotype						
CC	26 (76.5%)	6 (21.4%)				
CT	8 (23.5%)	22 (78.6%)	11.9 (3.6–39.6)	<0.001	6.4 (1.0–41.3) ^b	0.052

NOTE: Methylation-low corresponds to a PMR ≥ 0.01 in NCM and PBL. Tumors are defined as methylated when PMR ≥ 4.0 and as unmethylated when PMR < 4.0. Abbreviation: NT, not tested because of insufficient numbers.

^aOR adjusted for rs16906252 genotype.

^baOR adjusted for methylation-low in PBL and NCM.

The rs16906252 T allele is selectively methylated in normal tissues of colorectal cancer cases

To determine definitively whether *MGMT* methylation-low within normal tissues of colorectal cancer cases was attributable to selective methylation of the T allele, allelic-MSP encompassing rs16906252C>T (Fig. 2A), followed by clonal sequencing of the products, was performed in C/T heterozygotes who had exhibited methylation-low by qMSP. Among the 28 C/T cases in whom methylation-low had been detected in NCM and/or PBL, 20 NCM and 5 PBL samples from 24 cases were positive by allelic-MSP. The allelic-MSP products from 18 NCM and 4 PBL samples were successfully cloned and sequenced. As illustrated for representative samples (Fig. 2C), methylation was dense and monoallelic on the T allele in 17/18 NCM, and biallelic, but predominant on the T allele, in one NCM. Dense methylation specific to the T allele was observed in all 4 PBL (Fig. 2C).

Low-level *MGMT* methylation in PBL of healthy controls is associated with rs16906252C>T and occurs at similar frequency and levels as genotype-matched colorectal cancer cases

To determine whether *MGMT* methylation-low was detectable and associated with rs16906252C>T in the PBL of cancer-

free subjects, we screened 164 ARCBS controls of known genotype (134 C/C, 30 C/T) by qMSP. Methylation-low was detected in 11.9% of C/C and 50% of C/T subjects and was clearly associated with the T variant ($P < 0.0001$, Supplementary Table S4). When actual PMR values were analyzed as a continuous variable, methylation levels were higher in C/T (median, 0.01% ± 0.19%) than in C/C subjects (median, 0.00% ± 0.39%; $P < 0.0001$, Fig. 2D). The frequency and levels of methylation in PBL were similar in cases and controls when stratified by rs16906252C>T genotype (Supplementary Table S4; Fig. 2D). These findings indicate that low-level *MGMT* methylation in PBL is dependent on rs16906252C>T genotype and is unrelated to cancer status.

The rs16906252 T variant is associated with constitutively reduced transcription

To assess the effect of the T variant on *MGMT* promoter activity, we performed *in vitro* luciferase promoter reporter assays using constructs containing the *MGMT* promoter with either the wild-type C or the variant T allele at the rs16906252 site. In parallel transient transfections of the promoter reporter constructs, the T allele was expressed at levels of 65% and 63% relative to the C allele, in HEK293 and HCT116 cells, respectively (Fig. 3A).

(Continued.) Left, real-time qMSP assay was used to detect and measure methylation levels in subject-matched normal and tumor tissues. Right, allelic MSP assay was used to determine which allele of the rs16906252C>T SNP is methylated within normal tissues. **B**, Spaghetti plots showing PMR scores for *MGMT* methylation detected by qMSP in subject-matched PBLs, NCM, and CRC tissues from 62 CRC cases segregated by rs16906252 SNP genotype ($n = 32$ CC, $n = 30$ CT). Top, PMR scores for all three tissues, with values within subjects linked by a line. Bottom, expanded PMR scale showing the same values for PBL and NCM. **C**, Actual PMR values obtained by qMSP in the PBL of healthy controls and the same 62 CRC cases as (**B**), segregated by rs16906252C>T genotype. For **B** and **C**, P values are shown for statistical comparisons of PMR scores between groups, as indicated by a horizontal grey line. Comparisons of PMR scores for PBL or NCM between subjects across the groups of patients segregated by C/C and C/T genotypes were performed using the independent Mann-Whitney U test. Comparisons of PMR scores between subject-matched PBL and NCM were performed using a related samples Wilcoxon signed rank test. **D**, Clonal sequencing of allelic MSP products are shown for representative examples of normal tissues from rs16906252C>T heterozygous CRC cases in which low-level *MGMT* methylation was detected. Horizontal lines represent individual methylated alleles, and the methylation status of each CpG dinucleotide within each allele is shown by black and white circles. Top, NCM sample in which monoallelic methylation of the variant T allele (detected as A on the complementary strand) was detected, which was the most common pattern observed among the NCM of CRC cases. Bottom, the only NCM sample in which methylation of both alleles was detected, although more methylated T alleles than C alleles were detected.

To determine whether the T variant was associated with reduced expression *ex vivo*, the relative levels of transcripts derived from the C and T alleles of rs16906252C>T were measured in the NCM of rs16906252C>T heterozygous subjects, including 24 cases from the Sydney CRC series and 3 controls from St Vincent's Hospital, by allele quantification (AQ) RFLP at rs16906252 (Fig. 1C). Here, allelic expression levels were measured by densitometry of allele-specific bands post-electrophoresis (Supplementary Fig. S1A). AERs for the C allele relative to the T allele ranged from 1.4 to 4.8 (median, 2.3 \pm 0.83) in the cases and from 1.2 to 4.6 (median 1.7 \pm 1.84) in the controls, with no significant difference between cases and controls (Fig. 3B). Thus, a significant allelic expression imbalance (AEI) was observed between the two rs16906252 alleles in NCM, irrespective of cancer status, with expression from the T allele at 21% to 83% of that from the C allele.

To confirm these findings, we used an indirect AQ approach (Supplementary Fig. S1B). Using AQ pyrosequencing, the relative levels of allelic transcripts at a downstream reference SNP, rs1803965C>T located within exon 3, were measured in the NCM of a subset of 8 of the rs16906252C>T heterozygous cases who were also heterozygous for rs1803965C>T, and a comparison group of 9 rs1803965C>T heterozygous cases who were homozygous C/C at rs16906252 (Fig. 1C). Considerable variation in rs1803965C:T AERs were observed among the double-heterozygote group, with either genetic allele of the assayed rs1803965C>T SNP expressed at the higher level, whereas balanced allelic expression was observed among the cases homozygous C/C at rs16906252 (Supplementary Fig. S2). To adjust the AERs to reflect the transcriptional activity associated with rs16906252C>T in the double heterozygotes, we next determined the rs16906252; rs1803965 haplotypes by RT-PCR across exons 1 and 3 encompassing both SNPs, followed by clonal sequencing of the cDNA fragments (Supplementary Fig. S1B). The AERs for the double heterozygotes were then adjusted to rs16906252C:T AERs, irrespective of which allele showed higher expression at rs1803965 downstream. To minimize any statistical bias incurred by this adjustment, the rs1803965 AERs in the rs16906252 C/C homozygote group were adjusted to ≥ 1.0 , irrespective of which allele of this assayed SNP showed the highest expression level. The range of adjusted rs16906252C:T AERs in the double heterozygote group was 1.8–4.5 (median \pm SD: 2.5 \pm 0.78), indicating reduced expression associated with the rs16906252 T variant in all 8 cases (Fig. 3C). The adjusted AER values obtained for these 8 cases by AQ-RFLP and AQ pyrosequencing showed strong concordance (Supplementary Fig. S2C). The range of adjusted AERs among the rs16906252 C/C homozygotes was 1.08–1.54 (median \pm SD: 1.23 \pm 0.18; Fig. 3C). The degree of AEI in rs16906252C>T cases was significantly higher than in the C/C homozygotes ($P = 0.003$; Fig. 3C). The significant transcriptional reduction associated with the rs16906252 T variant was not inversely correlated with methylation levels in the NCM samples, therefore not a consequence of it (Fig. 3D). Collectively, these findings provide strong evidence that the rs16906252 T allele is constitutively expressed at a reduced level in NCM, irrespective of cancer status.

Discussion

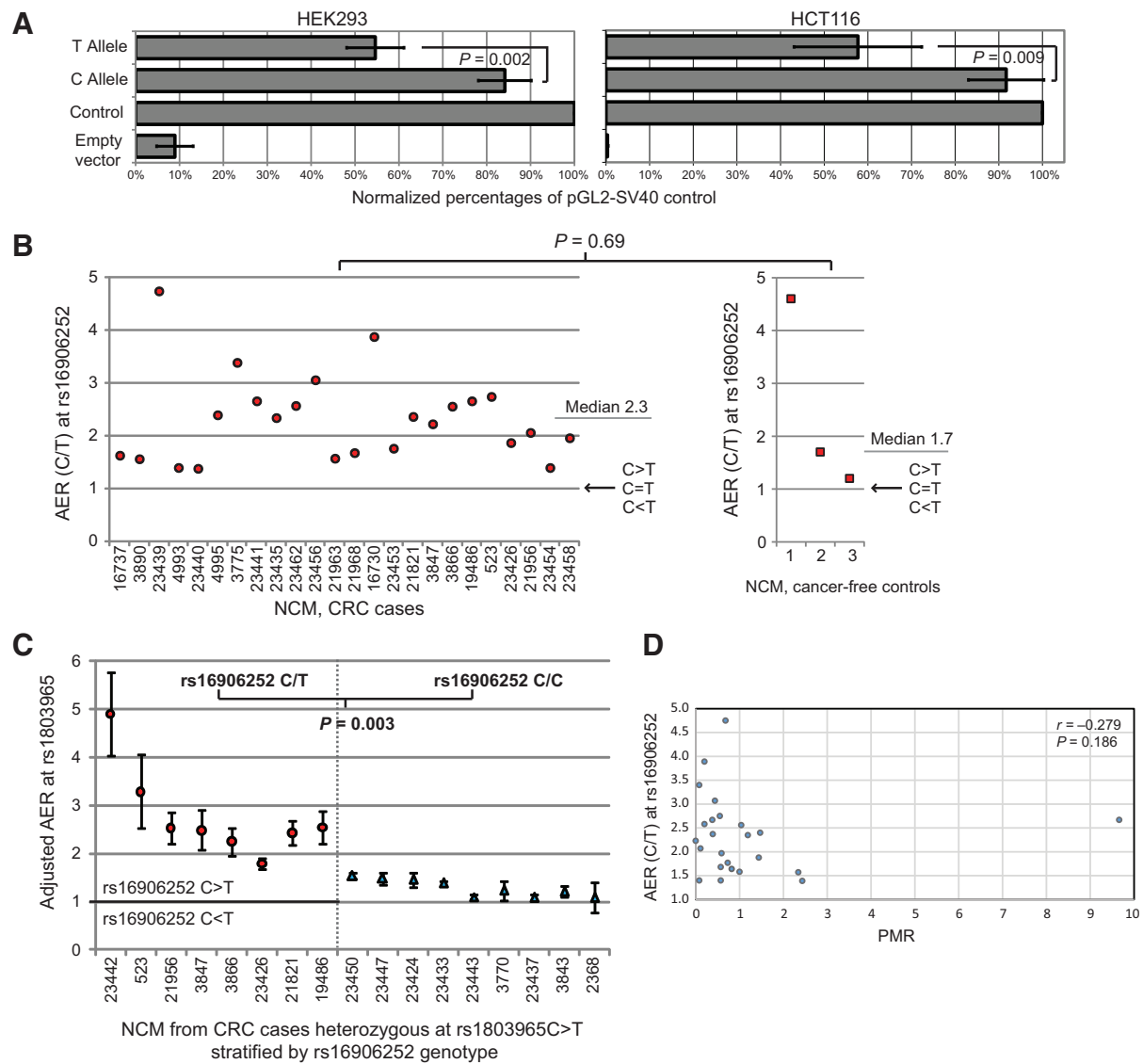
We firstly confirm the close association between rs16906252C>T genotype and *MGMT* methylation in primary colorectal cancer. Including our study, this association has now

been independently observed in 3 colorectal cancer series (2, 21). Similar findings at additional cancer sites suggest rs16906252C>T is a key determinant of *MGMT* methylation pan-cancer (8, 22–25).

A key novel finding of this study was evidence for an association between rs16906252C>T and an elevated risk of developing colorectal cancer exhibiting *MGMT* methylation, and conversely, a reduced risk of developing *MGMT*-unmethylated colorectal cancer. These risk associations were only identified by virtue of the molecular pathologic epidemiology approach taken, whereby risk associations between rs16906252C>T genotype and colorectal cancer was performed in a molecular subtype-specific manner after stratifying the cases by the *MGMT* methylation status of their tumor. This illustrates the power of molecular pathologic epidemiology, an emerging field that integrates pathology and epidemiology to take molecular heterogeneity of cancer into account in deciphering risk associations that may otherwise be imperceptible using standard molecular epidemiology approaches (34–37). The lack of an association between rs16906252C>T and overall risk for colorectal cancer is unsurprising, given that no cancer risk association has been reported for tag-SNPs around *MGMT* in genome-wide association studies (GWAS). rs16906252 is not represented on SNP arrays commonly employed for mass genotyping due to its low MAF in the general population, thus has not specifically been interrogated in GWAS of cancer.

One limitation of our case-control study was the lack of self-reported race/ethnicity data, hence for geographic origin matching purposes, our control groups were selected from the same catchment areas as the case series. The MAF reported for rs16906252C>T in dbSNP and the 1000 Genomes Project databases ranges from 4% to 9% for Europeans, 4% for South Asians, and 0% for Africans and East Asians. The MAFs of 6.1% to 6.3% in our controls and 7.0% to 7.5% in our cases is consistent with the high rate of Caucasians in the populations from which our subjects were recruited. A large prospective population-based study would be required to demonstrate whether the rs16906252C>T genotype predisposes to the development of cancers exhibiting *MGMT* deficiency.

In seeking to elucidate the mechanistic basis for the interaction between rs16906252C>T and risk for *MGMT*-methylated colorectal cancer, we detected low-level *MGMT* methylation in the PBL and NCM of colorectal cancer cases and in the PBL of healthy controls in association with rs16906252C>T. These findings are consistent with prior findings by our group in NCM from both colorectal cancer cases and controls (2) and by others in PBL from controls (26). Among colorectal cancer cases, genotype was a stronger predictor of an *MGMT*-methylated tumor than the presence of low-level methylation within either normal tissue. Nevertheless, among rs16906252C>T heterozygous cases, methylation levels were higher in NCM than in PBL, consistent with a role for this genotype-dependent *MGMT* methylation in field cancerization. Although global levels of methylation in these normal tissues were low, clonal sequencing revealed dense methylation of individual T alleles, consistent with methylation occurring in a small proportion of cells, predisposed by rs16906252C>T. The frequency and levels of *MGMT* methylation in PBLs did not differ between colorectal cancer cases and controls following stratification by rs16906252 genotype, further indicating that methylation was

**Figure 3.**

Reduced transcriptional activity from the T allele relative to the C allele of *MGMT* promoter enhancer SNP rs16906252. **A**, Luciferase promoter reporter assays show reduced promoter activity from the variant T allele. Bar charts of the *MGMT* promoter reporter activity produced by the HAP1 and HAP4 constructs containing the C allele or the T allele, respectively, at the rs16906252 SNP site are shown. Values are the mean \pm SD of firefly luciferase activity normalized to *Renilla* luciferase activity and expressed as a percentage of the pGL2-SV40 control vector from four independent experiments performed in triplicate. Cells transfected with the empty pGL2-basic vector show background levels of luciferase activity. *P* values determined by the Student *t* tests show the difference in expression levels between constructs containing the C and T alleles at the rs16906252 SNP site were significant in both cell lines. The T allele was expressed at 65% the level of the C allele in HEK293 cells, and 63% of the level of the C allele in HCT116 cells. **B**, Constitutively reduced expression from the rs16906252T allele compared with the C allele in NCM of heterozygous colorectal cancer (CRC) cases from the Sydney CRC series and controls without neoplasia, as detected by AQRFLP analysis at the rs16906252 SNP site (Supplementary Fig. S1A). AERs are plotted as rs16906252C:T. A C:T AER of 1.0 indicates equal levels of expression from the 2 alleles at the rs16906252 site. The AERs were consistently >1.0 , indicating the C allele is expressed at a higher level than the T allele. There was no significant difference in AERs between the CRC cases and cancer-free controls (Mann-Whitney *U* test). **C**, Reduced expression from the rs16906252T allele compared with the C allele in NCM of heterozygous colorectal cancer (CRC) cases from the Sydney CRC series. All cases were heterozygous for the rs1803965 exon 3 SNP at which allelic expression levels were determined by AQRFLP analysis (Supplementary Fig. S1B). Cases were then segregated by rs16906252 genotype into the heterozygous C/T group ($n = 8$, left) or the homozygous C/C group ($n = 9$, right). AERs plotted are the mean \pm SD from 3 replicates. Adjusted AERs for the double heterozygotes (left) are plotted as rs16906252C:T, irrespective of which allele of the assayed rs1803965 SNP was highest expressed. AERs for the rs16906252C/C homozygotes (right) were plotted as AER ≥ 1.0 , irrespective of which allele at rs1803965 was expressed at the highest level. The significant difference in the adjusted AERs from rs16906252C>T heterozygotes versus C/C homozygotes indicates the wild-type C allele at the rs16906252 is expressed at a significantly higher level than the minor T allele ($P = 0.003$; Mann-Whitney *U* test). Close concordance in results was observed for samples that were studied by both the AQRFLP and AQRFLP methods (Supplementary Fig. S1C). Unadjusted AER values are provided in Supplementary Fig. S2. **D**, Lack of correlation between the AER and levels of *MGMT* promoter methylation detected in the NCM of rs16906252C>T heterozygous CRC cases shows reduced expression from the T allele is not a consequence of low-level methylation. AER (rs16906252C:T) obtained by AQRFLP and PMR values obtained by qMSP are plotted for 24 CRC cases. Spearman ρ and *P* value are shown.

dependent on genotype, not cancer status or treatment. These findings provide firm evidence that rs16906252C>T represents a functional genetic variant that acts *in cis* to alter the somatic methylation state of the *MGMT* promoter, termed a methylation quantitative trait locus (meQTL). A recent study demonstrated a proportion of GWAS SNPs associated with cancer risk represent meQTLs, providing supportive evidence that SNP-associated secondary somatic epigenetic alteration may mediate cancer risk (38). We also demonstrated rs16906252 was associated with differential levels of allelic *MGMT* transcription, thus also represents an expression QTL (eQTL). Our luciferase promoter reporter assays showed that the T allele diminished promoter activity in HEK293 and HCT116 cells, consistent with prior findings in other cancer cell lines (8, 22), suggesting that rs16906252C>T directly effects transcriptional regulation of *MGMT*. More relevant, we showed that the T allele is expressed around half the level of the C allele in the NCM of both colorectal cancer cases and controls. This magnitude of transcriptional reduction was not attributable to *MGMT* methylation, which was present at low levels if at all, thus may be regarded as constitutive. Interestingly, rs16906252C>T predicted prolonged survival in glioblastoma patients treated with temozolomide, irrespective of whether their tumor was methylated or unmethylated at *MGMT* (8). Reduced transcription from the T allele provides one possible explanation for the extended survival experienced by T allele carriers whose glioblastoma was *MGMT*-unmethylated.

On the basis of our collective results, we propose a step-wise model to explain the risk association between rs16906252C>T and development of *MGMT*-methylated colorectal cancer. In this model, the T variant underlies constitutively reduced transcription (possibly by abrogating binding of transcriptional activators to the *MGMT* enhancer). This predisposes the T allele to chromatin remodeling, ultimately resulting in somatic promoter methylation within normal tissues. In turn, this stable epigenetic silencing predisposes to the development of *MGMT*-methylated cancer through field cancerization and clonal amplification of cells harboring *MGMT* methylation. The epigenetic alteration associated with rs16906252C>T within normal tissues likely serves as an intermediary linking this genotype with increased risk of developing an *MGMT*-methylated tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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