Detection of MGMT Promoter Methylation in Normal Individuals Is Strongly Associated with the T Allele of the rs1690625 MGMT Promoter Single Nucleotide Polymorphism

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Abstract
Methylation of the CpG island in the MGMT promoter region is a frequent event in several cancer types including colorectal cancer, lung cancer, lymphoma, and glioblastoma. A correlation between methylation and the T allele of the rs1690625 single nucleotide polymorphism (SNP) in colorectal carcinomas has previously been reported. As aberrant MGMT methylation can be an early event in tumor development, we tested the hypothesis that normal individuals possessing the T allele may be predisposed to somatic methylation at the MGMT promoter. Peripheral blood mononuclear cell DNA from 89 normal, healthy individuals was genotyped at rs1690625 and assessed for the methylation status of the MGMT promoter region using independent quantitative methodologies capable of detecting low-level methylation: MethyLight and Sensitive Melting Analysis after Real-time Methylation-Specific PCR (SMART-MSP). There was a strong association between presence of the T allele and detectable methylation \( P = 0.00005 \) in the peripheral blood DNA. Furthermore, when a MSP assay flanking the SNP was used to amplify methylated sequences in heterozygotes, only the T allele was methylated. Thus, detectable somatic methylation of the MGMT promoter in normal individuals is strongly associated with the T allele of the rs1690625 MGMT promoter SNP.

Inactivation of tumor suppressor genes and DNA stability genes by promoter methylation is a common occurrence in human cancer. In some cases, epigenetic inactivation of one of these genes is likely to be the initiating event in cancer development (reviewed in ref. 1). Evidence for this is particularly strong for certain hereditary cancer genes where constitutional methylation of the gene predisposes to cancer development (2–4).

It is also likely that constitutional methylation of other genes not normally involved in heritable cancer can predispose to cancer. One of the most plausible candidates for a gene whose inactivation may initiate carcinogenesis is MGMT, which codes for a protein that removes alkyl adducts from the O6 position of guanine (5). Loss of MGMT function will give rise to a mutator phenotype, as alkyl damage from a variety of environmental sources is a common occurrence and as alkylated guanine is likely to mispair with thymine during DNA replication. MGMT methylation is commonly found in various cancers including colorectal cancers, gliomas, head and neck cancers, and lymphomas (6). MGMT promoter methylation has been used as a predictive marker in cancers; it indicates those individuals who are likely to respond to chemotherapy with alkylating agents (7, 8).

MGMT methylation may be an early or even predisposing event in colorectal cancer. Esteller et al. (9) reported that MGMT methylation was present in adenomas. Shen et al. (10) reported that MGMT methylation was found in apparently normal colonic tissue up to 10 cm from an MGMT-methylated colorectal cancer indicating that MGMT methylation can even be observed prior to any detectable change in morphology.

Ogino et al. (11) investigated whether single nucleotide polymorphisms (SNP) in the MGMT gene had an effect on methylation of MGMT in colorectal cancers. The T allele of a SNP within the 5′ untranslated region (UTR; rs16906252; c.-56 C>T) was strongly associated with promoter methylation. We reasoned that the T allele might also affect the propensity to methylate the MGMT promoter in normal individuals. We thus sought to determine if mosaic methylation at the MGMT promoter was present in a readily assayed tissue, the peripheral blood, and if this was associated with the T allele.
Materials and Methods

Samples

The investigations were done after approval by the Peter MacCallum Cancer Centre Ethics Committee (Project 02/70). Eighty-nine peripheral blood samples from normal blood donors were obtained after informed consent from the Australian Red Cross Blood Service.

DNA extraction and treatment

Mononuclear cells were extracted from peripheral blood using Histopaque-1077 (Sigma Aldrich) as per the manufacturer’s instructions, and DNA was extracted using the Puregene kit (Qiagen) omitting the red cell lysis step, or using the salting out method (12). DNA quantitation was done on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). DNA was diluted to 5 ng/μL in DNA hydration solution (Qiagen) for SNP genotyping.

Bisulfite modification was done on 1 μg DNA with the MethylEasy Xceed kit (Human Genetic Signatures) according to the manufacturer’s instructions, using two elutions of 50 μL, giving a final theoretical concentration of 10 ng/μL. For some samples, 500 ng of DNA were modified using the 96-well Epitect kit (Qiagen) eluting in a final volume of 40 μL. For DNA modified with MethylEasy Xceed, 1 μg Universal methylated DNA (Chemicon) was used as the unmethylated control and second round whole genome amplified product was used as an unmethylated control. For Epitect modified DNA, methylated and unmethylated DNA controls from Qiagen were used.

Genotyping by high resolution melting analysis

The primers used for genotyping the rs16906252 SNP were 5′-CTTTGCGTCCGGCCGCAG-3′ and 5′-CCAGAAGACT-CAACCAAGTTCCGGA-3′. PCR cycling and HRM was done on the Rotor-Gene Q (Qiagen) in 100 μL PCR tubes with a final volume of 20 μL, containing 200 mmol/L of each primer, 200 mmol/L of each deoxynucleotide triphosphate, 0.5 units of HotStarTaq DNA polymerase (Qiagen) in the supplied PCR buffer containing 2.5 mmol/l MgCl₂, 5 mmol/L SYTO9 (Invitrogen), and 10 ng of bisulfite-treated DNA.

The initial denaturation (95°C, 15 min) was followed by 50 cycles of 20 s at 95°C, 30 s at 68°C (67°C for addition of m13 tags before sequencing), 30 s at 72°C; 1 cycle of 1 min at 95°C, 72°C for 1.5 min, and an HRM step from 65°C to 90°C increasing at 0.2°C per second, and holding for 1 s after each stepwise increment. Five nanograms of DNA were used as template for genotyping, and either 10 or 12.5 ng DNA were used for methylation studies. All reactions were done in duplicate.

Sequencing was done using Big Dye Terminator v3.1 chemistry (Applied Biosystems) according to the manufacturer’s instructions. The initial denaturation (96°C, 1 min) was followed by 30 cycles of 10 s at 95°C, 5 s at 50°C, and 4 min at 60°C. The sequencing products were purified by ethanol precipitation and separated on a 3100 Genetic Analyser (Applied Biosystems).

Results

A panel of 89 normal individuals (Red Cross blood donors) were genotyped for the rs16906252 SNP. We detected 77 CC homozygotes, 11 CT heterozygotes, and 1 TT homozygote.

DNA prepared from the mononuclear cell fraction of peripheral blood was tested for MGMT methylation by two sensitive methodologies capable of detecting low-level methylation: SMART-MSP and MethyLight. SMART-MSP is an methylation-specific PCR (MSP)-based methodology that uses real-time amplification for quantification and high resolution melting analysis (HRM) to quality control the results (14). This allowed us to exclude false positive results due to incomplete conversion. MethyLight is an MSP-based methodology that uses a TaqMan probe to quantify the results and to select against false positive amplification (15).

The SMART-MSP assay region was located in the 5′ UTR, immediately upstream of the rs16906252 SNP and is shown in Fig. 1. The TT homozygote, 6 of the 11 CT heterozygotes and 3 of the 77 CC homozygotes showed mosaic methylation using the

Fig. 1. Map of the MGMT promoter showing the location of primers and probes. The sequence shown starts at the transcription start site, continues through the proximal 5′ UTR into the beginning of the first intron. Vertical lines indicate CpG dinucleotides. * indicates the position of the SNP. The positions of the primers (arrows) flanking the SMART-MSP and MethyLight amplicons are indicated as well as the position of the MethyLight probe (l-l).
assay \((P > 0.0001; \text{Table 1A})\). The levels of methylation ranged from 0.1% to 9.7% (Table 2). The level of methylation detected in the three CC individuals was low (0.1% or 0.2%). Very similar results were obtained using a MethyLight assay (Table 1B; Fig. 2C). The region assayed and its relationship to the SMART-MSP assay is shown in Fig. 1. None of the CC homozygotes had detectable methylation using the MethyLight assay (Table 1B).

To determine whether there was preferential methylation of either of the alleles in the heterozygous individuals, a second SMART-MSP assay was designed using primers that flanked the rs16906252 SNP (Fig. 1). The assay was targeted to the antisense strand containing the A/G alleles, as the C/T SNP is lost after bisulfite conversion converts the C to a T in the sense strand. This assay gave methylation results that were very similar to the first assay and the MethyLight assay, although there was variation in the estimated level of methylation (Tables 1C and 2; Fig. 2).

This second SMART-MSP assay containing the SNP allowed us to determine which allele was methylated as only methylated alleles were amplified. HRM showed that all six heterozygotes with detectable levels of promoter methylation displayed methylation of the T allele only (data not shown). This result was confirmed by bisulfite sequencing of the antisense strand of a longer MSP amplicon that also included the SNP (amplicon shown in Fig. 1). All products of the heterozygotes showed only the A allele confirming that only the T allele was methylated in all of the heterozygotes (Figs. 2E and 3).

### Discussion

The mechanisms underlying the inheritance of epigenetic alterations are currently unclear. Although there is some evidence that not all epigenetic marks are erased in the germline (16) in other cases, it is increasingly clear that rare or common sequence alterations underlie the propensity to epigenetic change. Most of the cases described involve rare sequence changes (17, 18).

In this article, we examined methylation of the MGMT gene in normal peripheral blood tissues in relation to the rs16906252 SNP. Our reasons for doing this were two fold. First, it had been shown that there was a strong relationship between the T allele of this SNP in the MGMT 5′ UTR and the methylation of this gene in the tumors of patients with colorectal cancer (11). Second, Shen et al. (10) had reported that 80% of colorectal cancer patients that had MGMT methylation in their tumors also had MGMT methylation in colonic mucosa >10 cm away from the tumor. It was proposed that a “field defect” consistent with a precancerous lesion that already had MGMT inactivated was responsible for this phenomenon.

We hypothesized that the SNP genotype could influence methylation in normal tissues and chose the peripheral blood as a completely unrelated normal tissue in which to examine this hypothesis. In accordance with our hypothesis, MGMT methylation was significantly associated with the T allele of the SNP. The independent sensitive assays used by us gave near concordant results. The levels in most cases were sufficiently low so that they would have been either not observed or dismissed as artifacts by most methodologies. Furthermore, only the T allele was methylated in the heterozygotes supporting its susceptibility to methylation.

### Table 1. Summary of data

<table>
<thead>
<tr>
<th>Sample Genotype</th>
<th>Methylation</th>
<th>Unmethylation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Summary of data from SMART-MSP assay 1*</td>
<td>3</td>
<td>74</td>
<td>77</td>
</tr>
<tr>
<td>CC</td>
<td>3</td>
<td>74</td>
<td>77</td>
</tr>
<tr>
<td>CT and TT</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>79</td>
<td>89</td>
</tr>
<tr>
<td>B. Summary of data from MethyLight assay†</td>
<td>0</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>CC</td>
<td>0</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>CT and TT</td>
<td>4§</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>4§</td>
<td>80</td>
<td>84</td>
</tr>
<tr>
<td>C. Summary of data from SMART-MSP assay 2 (SNP flanking)</td>
<td>2</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>CC</td>
<td>2</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>CT and TT</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>80</td>
<td>89</td>
</tr>
</tbody>
</table>

*Fisher’s test \(P = 3.7 \times 10^{-5}\).

†Fisher’s test \(P = 1.17 \times 10^{-5}\).

§Five are missing because they were not done — insufficient DNA.

‡Two not included came up as false positives.

### Table 2. Concordance of methylation data across experiments

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype</th>
<th>SMSP</th>
<th>SMSP (SNP)</th>
<th>MethyLight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CC</td>
<td>0.2%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>CC</td>
<td>0.1%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>CC</td>
<td>0.1%</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>CC</td>
<td>–</td>
<td>0.2% G</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>CC</td>
<td>–</td>
<td>0.1% G</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>TT</td>
<td>0.1%</td>
<td>3.1% A</td>
<td>0.2%</td>
</tr>
<tr>
<td>7</td>
<td>CT</td>
<td>9.7%</td>
<td>4.5% A</td>
<td>3.1%</td>
</tr>
<tr>
<td>8</td>
<td>CT</td>
<td>8.4%</td>
<td>2.5% A</td>
<td>0.2%</td>
</tr>
<tr>
<td>9</td>
<td>CT</td>
<td>0.8%</td>
<td>0.1% A</td>
<td>1.2%</td>
</tr>
<tr>
<td>10</td>
<td>CT</td>
<td>0.4%</td>
<td>1.7% A</td>
<td>0.2%</td>
</tr>
<tr>
<td>11</td>
<td>CT</td>
<td>0.2%</td>
<td>0.1% A</td>
<td>0.1%</td>
</tr>
<tr>
<td>12</td>
<td>CT</td>
<td>0.1%</td>
<td>0.1% A</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>CT</td>
<td>–</td>
<td>0.8% A</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>CT</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>CT</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>16</td>
<td>CT</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

NOTE: The results of three separate assays are summarized. All the CT and TT samples are shown. In addition, all CC samples that displayed methylation are shown. The SMSP column shows results from the first SMART-MSP assay, the SMSP (SNP) column shows results from the second SMART-MSP assay that contains the SNP. The column also shows the allele (antisense) detected by HRM and sequencing.

Abbreviations: –, no methylation detected; ND, not done.
A minority (5 of 77) of CC homozygotes showed very weak methylation in one or other of the SMART-MSP assays. This argues that other factors also influence the methylation of the \( MGMT \) promoter and is consistent with the methylation of the C allele in cancer. In addition, the observation that some of the CT heterozygotes showed undetectable or very weak methylation, whereas other CT heterozygotes showed higher levels of methylation also argues that other factors also influence the methylation of the \( MGMT \) promoter.

The phenomenon reported here is distinct from a field defect as it involves an unrelated tissue. However, one would expect the association of \( MGMT \) methylation with the T allele of the SNP to be present in all tissues, even if there is no significant selective advantage of \( MGMT \) methylation, thus giving rise to a constitutional methylation epigenotype. We thus would predict that these individuals would also show methylation in their colonic mucosa and if this was the only tissue examined, would be ascribed to a field defect. Whether the methylation affects a higher proportion of cells and whether these methylated cells are at greater risk of becoming cancerous remains to be determined.

Another example of a common variation affecting epimutations is a 12-bp insertion polymorphism at the \( RIL \) gene.
shown that the insertion created an additional Sp1/3 binding site that conferred some protection against methylation for this allele in normal colon (19). There are, however, significant differences between our results and those reported for the RIL gene. RIL shows strong aging dependent methylation, whereas this was not seen in our study (data not shown). Second with RIL, the long variant confers only partial protection against methylation and both alleles become methylated in normal tissues albeit at different frequencies.

This is the first study showing the association of a SNP in a cancer predisposition gene with propensity to methylation of that gene in normal individuals. Kerckel et al. (20) recently showed that for a number of SNPs, the methylation shows a strong predilection to one allele. However, the methodology used identified strongly methylated alleles, whereas this study show that the methylation changes associated with particular SNP alleles may be considerably more subtle.

There has been some debate regarding the inheritance of epimutations. Do constitutional epimutations arise from inheritance of sequence variants resulting in predisposition to epimutations or is there actual inheritance of epimutations (21–24)? Whereas there are examples where epimutations do seem to be inherited (16), in most cases, a genetic element influencing epigenetic alteration in cis is a more likely explanation.

Our study shows that for MGMT, the T allele of the rs16906252 SNP, underlies the predisposition to methylate in cis. Whether this allele predisposes to cancer is still unclear at this stage. If it does do so, the possibility of identifying individuals at risk of cancer either by genotyping for the SNP or by assaying for peripheral blood methylation becomes a possibility.

Disclosure of Potential Conflicts of Interest

A. Dobrovic is an inventor of a provisional patent for the Sensitive Melting Analysis after Real-time Methylation-Specific PCR methodology used in this article. The other authors disclosed no potential conflicts of interest.

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