Silencing of O^6-methylguanine DNA methyltransferase in the absence of promoter hypermethylation in hepatocellular carcinomas from Australia and South Africa

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Abstract. The DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) is involved in cellular defences against alkylating agents. Alterations in the *MGMT* gene may result in an increase in the mutation rate and risk of malignant transformation. We have previously shown that MGMT is implicated in colorectal carcinogenesis particularly in cancers which display microsatellite instability, a marker of impaired DNA repair. The aims of the current study were to assess the roles of *MGMT* and microsatellite instability in hepatocellular carcinomas (HCCs) from Australia and South Africa. DNA was extracted from malignant and non-malignant liver tissue from 37 Australian and 24 South African patients, and histologically normal liver from 20 transplant donors. *MGMT* promoter hypermethylation and MGMT protein expression were assessed using methylation specific PCR and immunohistochemistry. Microsatellite instability was examined using a panel of 23 microsatellite markers previously used to detect allelic imbalance and two specific markers for the detection of low levels of microsatellite instability. Methylation specific PCR did not detect any methylation of the *MGMT* promoter in Australian and South African HCCs. Similarly, no hypermethylation of *MGMT* was observed in the adjacent non-malignant liver or histologically normal liver. MGMT staining was predominantly nuclear with some cytoplasmic staining. Overexpression of MGMT protein was detected in 14 (39%) HCCs, while a reduction in protein expression was evident in 14 (39%) HCCs. In the remaining 8 cases the expression of MGMT was comparable in HCCs and adjacent non-malignant tissue. Interestingly, MGMT expression decreased relative to adjacent non-malignant liver tissue in patients who had aetiologies other than viral hepatitis for their underlying liver diseases ($p<0.02$). No microsatellite instability was detected in this series of 61 HCCs. This suggests that epigenetic silencing of *MGMT* and microsatellite instability does not play an important role in this series of HCCs derived from different populations.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with an estimated annual incidence of one million cases (1,2). Various genetic and epigenetic abnormalities have been identified in HCCs, however hepatocarcinogenesis is poorly understood at the genomic level.

O^6-methylguanine DNA methyltransferase (*MGMT*) is a DNA repair protein that removes mutagenic and cytotoxic adducts from the O6 position of guanine (3). O^6-methylguanine is a powerfully pro-mutagenic DNA base lesion which, if not repaired, can lead to G-A transition mutations (3). This has been implicated in the activation of the malignant H-ras oncogene and disturbance of the p53 tumour suppressor function (3). Animal models and *in vitro* studies have demonstrated that a deficiency in MGMT repair is a major determinant of susceptibility to carcinogenesis by alkylating agents (4).

Collier *et al* examined MGMT enzyme activity in normal, cirrhotic and non-cirrhotic livers with chronic liver disease and observed a significant reduction of MGMT levels in cirrhotic tissue compared to non-cirrhotic diseased liver and normal liver (5). These findings implicate loss of MGMT function in the pathogenesis of HCC. Inactivation of *MGMT* has been demonstrated in a range of malignancies (6-10).

Recent studies have demonstrated that promoter hypermethylation may result in inactivation of *MGMT* as this is associated with loss of MGMT expression in primary human malignancies such as gliomas, lymphomas and colon carcinomas (6-10).

Microsatellites are short (1 to 5 bases) tandem repeats scattered throughout the genome. They are prone to strand slippage during DNA replications. Failure to repair these slippages results in a change in the number of repeats and the size of the microsatellite allele. This microsatellite instability (MSI) is a phenotypic marker of defective DNA mismatch repair. In cancers with defective mismatch repair, MSI will affect a third or more of all mono- and di-nucleotide microsatellites (11). These cancers are designated MSI-high (MSI-H). Fifteen percent of sporadic colorectal carcinomas (CRCs) are MSI-H, while 70-75% have no evidence of MSI and are considered microsatellite stable (MSS) cancers. The remaining 10% of sporadic CRCs appear to have low levels of MSI (MSI-L), affecting predominantly the higher order microsatellite repeats (11,12). Recently it has been suggested that silencing of the *hMLH1* gene by promoter hypermethylation is the mechanism underlying the presence of the MSI phenotype in sporadic CRCs.

In the liver, both MGMT and MSI have been implicated in hepatocarcinogenesis, although the degree of MSI in HCCs, if present, has been less than typically seen in malignancies with known impairment of DNA mismatch repair (13-17). We have previously shown an association between *MGMT* promoter hypermethylation, reduced MGMT protein expression and MSI-L in sporadic CRCs (10). The relationship between MGMT and MSI in hepatic carcinogenesis has not been explored. The aim of the current study was to determine the extent of *MGMT* promoter hypermethylation, protein expression, and microsatellite instability and the relationship between these in a cohort of 61 Australian and South African HCCs.

Materials and methods

Subjects. Malignant and adjacent non-malignant liver tissue was obtained during surgery from 37 Australian (28 Caucasian and 9 Asian) and 24 male black South African patients, and frozen at -70˚C until DNA extraction was performed. The M:F sex ratio of the Australian patients was 28:9 and the median age was 61 years (range: 28-77 years). The median age of the South African patients was 35 years (range, 23-67 years). In addition, samples of histologically normal liver tissue from 20 transplant donors whose families had consented to this tissue being used for scientific research purposes were also studied. There were 12 males and 8 females in the donor group with a median age of 38 (range: 14-71 years). Additional clinical data are shown in Table I. This study was approved by the Human Research Ethics Committees of The University of Queensland and The Queensland Institute of Medical Research.

Methylation specific PCR (MSP). The methylation status of *MGMT* was determined by methylation specific PCR (MSP) using six CpG sites within the promoter region of this gene (18). This sensitive technique is based on the observation that unmethylated cytosines in bisulfite-modified genomic DNA are converted to uracil bases, whereas methylated cytosines are preserved. The region of interest is then amplified with primers specific to either the methylated DNA or to the modified DNA sequence corresponding to unmethylated DNA. The PCR products are visualised on a 10% non-denaturing polyacrylamide gel stained with ethidium bromide. This method and primer set has been successfully used to detect *MGMT* promoter hypermethylation in other cancers (18,19). Additional assay details have been published previously (10,18,19). DNA from colon cancer tissue previously shown to have *MGMT* promoter hypermethylation was used as the positive control.

Immunohistochemistry. Paraffin sections were affixed to Superfrost plus adhesive slides (Menzel-Glaser, Braunschweig, Germany). After dewaxing and rehydration to distilled water, the sections were subjected to heat antigen retrieval in 0.001 M EDTA (pH 8.0). Endogenous peroxidase activity in sections was blocked using 1.0% H_2O_2, 0.1% NaN_3 in TBS [(0.05 M Tris and 0.15 M NaCl (pH 7.2-7.4)]. Once transferred to a humidified chamber, the sections were incubated with 10% normal (non-immune) goat serum (Zymed Corp., San Francisco, CA). The sections were incubated overnight with a mouse anti-MGMT monoclonal antibody (clone MT3.1; Neomarkers, Fremont, CA) and diluted at 1:125 in TBS. After washing in TBS, biotin-like activity was blocked using the Biotin blocking kit (Dako Corp., Carpenteria, CA). The sections were subsequently incubated with biotinylated goat anti-mouse immunoglobulins (Jackson ImmunoResearch, West Grove, PA) at 1:400 dilution and then with streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch) diluted at 1:600. Colour was developed in 3,3'-diaminobenzidine (Sigma Chemical Co., St Louis, MO) with H_2O_2 as a substrate. Normal epithelium and stromal cells provided a positive internal control.

MGMT immunohistochemical stains of each tumour sample were graded independently by two observers (MDW, JRJ) and discordances resolved by consensus. MGMT stains were scored as previously described (10), briefly: (1) negative or trace amounts, (2) less than stromal cells (weak expression), (3) same as stromal cells (moderate expression) and (4) more than stromal cells (strong expression). Formalin-fixed tissue was not available from South African patients and frozen tissue could not be stained for MGMT due to the presence of freezing artefact.

Microsatellite instability. MSI was assessed with a panel of 23 dinucleotide microsatellite markers: D1S243, RIZ CA repeat, RIZ polymorphism (1p); D4S2983 (4p); D4S1538, D4S406, D4S426 (4q); 8p, D8S261, 8ptetra (8p); D9S162, D9S171, D9S1752, p14^{ARF}, D9S1748, p16^{INK4a}, D9S1604, D9S157 (9p); D13S1315 (13q); D16S347, D16S496 (16q); and p53ivs and p53VNTR (17p), used previously to detect allelic imbalance. In addition, the microsatellite instability status of all 61 HCCs was analysed using the tetranucleotide marker, MYCL, located at 1p32 and the dinucleotide marker D2S123 located on 2p16, recommended by the National Cancer Institute workshop for the detection of MSI-L cancers (20). Primer sequences were obtained from the Genome

Table I. Summary of patient clinical details, MGMT methylation, protein expression and MSI.

Australian HCCs[a]	Risk factors for HCCs[c]	Cirrhosis	O^6-MGMT methylation and microsatellite instability	O^6-MGMT immunohistochemistry[d]
1	Alcohol	No	No	1
2	Nil	No	No	2
3	NA	No	No	3
4	Haemochromatosis[b]	Yes	No	2
5	HCV	N/A	No	N/A
6	Haemochromatosis[b]	Yes	No	4
7	HBV	Yes	No	4
8	Haemochromatosis[b]	No	No	4
9	Allagile's	Yes	No	4
10	Nil	No	No	1
11	HBV	No	No	4
12	Nil	No	No	1
13	Nil	No	No	4
14	Nil	No	No	3
15	Cryptogenic	Yes	No	3
16	HBV	Yes	No	4
17	HCV/HBV	Yes	No	4
18	Alcohol	Yes	No	4
19	Haemochromatosis[b]	Yes	No	3
20	HBV	Yes	No	4
21	HBV	No	No	4
22	HCV + alcohol	Yes	No	2
23	Nil	No	No	2
24	HCV	Yes	No	4
25	Alcohol	Yes	No	2
26	Haemochromatosis[b]	Yes	No	2
27	Haemochromatosis[b]	Yes	No	3
28	Haemochromatosis[b]	No	No	3
29	Haemochromatosis[b]	Yes	No	2
30	Alcohol	Yes	No	2
31	Alcohol	Yes	No	2
32	Haemochromatosis[b]	No	No	3
33	HCV	Yes	No	2
34	Alcohol	Yes	No	4
35	HBV	No	No	3
36	HCV	Yes	No	4
37	HCV	No	No	1
38	HBV + aflatoxin	Yes	No	N/A
39	HBV + aflatoxin	Yes	No	N/A
40	HBV + aflatoxin	Yes	No	N/A
41	HBV + aflatoxin	No	No	N/A
42	HBV + aflatoxin	No	No	N/A
43	HBV + aflatoxin	Yes	No	N/A
44	HBV + aflatoxin	Yes	No	N/A
45	HBV + aflatoxin	No	No	N/A
46	HBV + aflatoxin	Yes	No	N/A
47	HBV + aflatoxin	No	No	N/A
48	HBV + aflatoxin	No	No	N/A
49	HBV + aflatoxin	Yes	No	N/A

Table I. Continued.

Australian HCCs[a]	Risk factors for HCCs[c]	Cirrhosis	O^6-MGMT methylation and microsatellite instability	O^6-MGMT immunohistochemistry[d]
50	HCV	No	No	N/A
51	HBV	Yes	No	N/A
52	HBV + aflatoxin	Yes	No	N/A
53	HBV + aflatoxin	No	No	N/A
54	HBV + aflatoxin	No	No	N/A
55	HBV + aflatoxin	No	No	N/A
56	HBV + aflatoxin	No	No	N/A
57	HBV + aflatoxin	No	No	N/A
58	HBV + aflatoxin	No	No	N/A
59	HBV + aflatoxin	Yes	No	N/A
60	HBV + aflatoxin	No	No	N/A
61	HBV	No	No	N/A

[a]Patients 1 to 37 were Australian while cases 38 to 61 were black South African. [b]All patients with haemochromatosis had been de-ironed prior to diagnosis of HCC. [c]HBV and HCV refer to chronic hepatitis B and C infection respectively. [d]Grading of O^6-MGMT protein expression: 1, negative or trace amounts; 2, weak expression; 3, moderate expression; 4, strong expression.

Database (http://gdbwww.gdb.org/) or from previous studies (21-23). Each microsatellite marker was amplified by PCR in a final volume of 15 μl incorporating 1.5 μCi of α^{33}P dATP. The amplified products were electrophoresed on 5% denaturing polyacrylamide gels and visualised by autoradiography.

Statistical analysis. Chi-squared tests (where asymptotic assumptions were satisfied) or Fisher's exact tests (for small groups) were used to compare the prevalence of promoter methylation and associated risk factors, where appropriate. A p-value of <0.05 was considered statistically significant.

Results

Amongst the 37 HCCs from Australia, 9 had haemochromatosis, 6 were positive for hepatitis C virus (HCV), 6 for hepatitis B virus (HBV), one for both HBV and HCV, 6 patients presented with a history of excess alcohol and one patient had Allagille's syndrome. Eight Australian patients had no known underlying liver disease. Evidence of cirrhosis was seen in 21 of 37 Australian HCCs. Among the South African patients, 23 had a history of HBV infection, 21 of these also with dietary aflatoxin exposure. One South African patient had chronic HCV. Cirrhosis was present in 10 South African patients.

MGMT promoter methylation in DNA from 61 primary HCCs, adjacent non-malignant tissue and 20 histologically normal tissues were analysed by MSP. The region studied is the area of greatest CpG density in *MGMT* and is immediately 5' to the transcription start site, an area previously found to be hypermethylated in a number of malignancies (9). MSP did not detect any methylation of the *MGMT* promoter in Australian and South African HCCs. Similarly, no hypermethylation of the *MGMT* promoter was observed in the adjacent non-malignant liver or histologically normal liver. Information regarding underlying liver disease, the presence of cirrhosis, microsatellite instability, *MGMT* methylation and MGMT protein expression is summarised in Table I.

No MSI was detected with the panel of 23 microsatellite markers or the two markers (D2S123, MYCL1) used to assess low levels of MSI in this series of 61 HCCs.

Thirty-six Australian HCCs were examined for MGMT protein expression using immunohistochemistry. When present, MGMT staining was predominantly nuclear with weak to moderate cytoplasmic staining. Overexpression of MGMT protein was detected in 14 (39%) HCCs, while a reduction in protein expression was evident in 14 (39%) HCCs (Fig. 1). In the remaining 8 cases the expression of MGMT was similar in HCCs and adjacent non-malignant tissue. Stromal expression was variable but maintained in all non-malignant liver tissue. Immunohistochemistry data are summarised in Table I.

This study found no association between viral hepatitis and MGMT expression, rather MGMT expression decreased relative to adjacent non-malignant liver tissue in patients who had aetiologies other than viral hepatitis for their underlying liver disease ($p<0.02$). However, the number of patients with viral hepatitis in the Australian group was small. No significant correlations were identified between MGMT expression and other clinical parameters.

Discussion

In cell culture, a causal relationship has been demonstrated between methylation of *MGMT* and transcriptional silencing of this gene (6-8). Furthermore, a number of malignancies show a tight correlation between *MGMT* CpG island methylation and loss of protein expression (24-29). However,

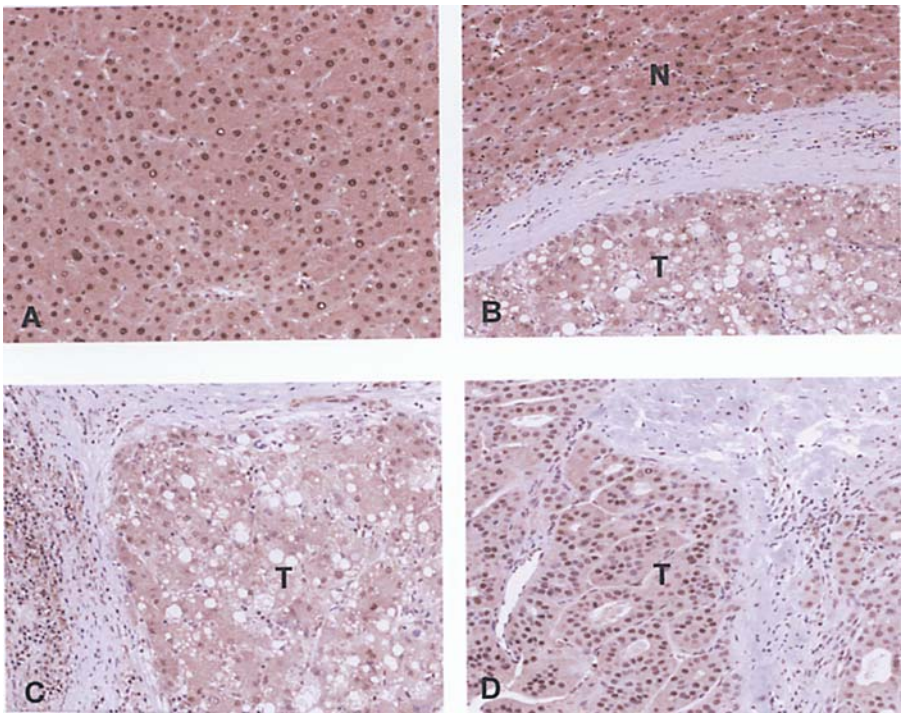

Figure 1. Immunohistochemistry for O^6-methylguanine methyltransferase. (A) Normal hepatocytes showing intense nuclear MGMT staining and moderate cytoplasmic staining (x20). (B) Junction of malignant (T) and non-malignant (N) liver showing reduced staining in the HCC relative to normal liver (x20). (C) HCC demonstrating heterogeneous reduction or complete loss of MGMT (x20). Stromal cells retain strong expression. (D) HCC showing intense nuclear and cytoplasmic staining for MGMT (x20).

Esteller *et al* reported that *MGMT* hypermethylation resulting in decreased MGMT protein expression is not universal in human malignancies such as breast, ovarian carcinoma and meningioma (24,25,29).

We have previously reported hypermethylation of *MGMT* in a subset of colon cancers using this MSP technique (10). This study also demonstrated a strong association between methylation of *MGMT* and a low level of microsatellite instability in sporadic colon carcinomas. The current study did not detect epigenetic alterations of the *MGMT* promoter in a cohort of 61 Australian and South African HCCs. Furthermore, no MSI was detected in our cohort of 61 HCCs, supporting data from previous studies (14,16,30). The absence of *MGMT* hypermethylation using this previously published assay does not appear to be due to failure of the technique as appropriate results were detected with the positive controls for MSP. Based on these data we have to conclude that methylation of *MGMT* did not appear to play a role in this series of HCCs.

The loss of MGMT protein expression observed in a subset of HCCs in the current study suggests that a mechanism other than promoter hypermethylation of the CpG sites studied is involved in the silencing of this gene. Supporting this observation, a subset of tumours displaying reduced protein expression in the absence of promoter hypermethylation has previously been detected in CRCs, implicating other mechanisms of inactivation (10).

Matsukura *et al* demonstrated an association between the promoter methylation status and viral infection, suggesting that hepatitis viral infections could play a role in the CpG methylation of the *MGMT* promoter (31). The current study has different findings with a statistically significant association between reduced MGMT expression and the absence of viral hepatitis in the Australian group. All 24 South African patients with HCCs had underlying viral hepatitis, however, the study of MGMT expression in these cases was not possible due to a lack of suitable tissue for immunohistochemistry.

Our data suggest that epigenetic silencing of *MGMT* and microsatellite instability does not play a role in Australian and South African HCCs. Nonetheless, the mechanisms underlying the reduced MGMT expression in a subset of these cancers is worth further investigation. A prospective study of a larger cohort is warranted to explore these observations.

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