

## Relationship between promoter methylation & tissue expression of *MGMT* gene in ovarian cancer

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**Background & objectives:** Epigenetic alterations, in addition to multiple gene abnormalities, are involved in the genesis and progression of human cancers. Aberrant methylation of CpG islands within promoter regions is associated with transcriptional inactivation of various tumour suppressor genes. O<sup>6</sup>-methylguanine-DNA methyltransferase (*MGMT*) is a DNA repair gene that removes mutagenic and cytotoxic adducts from the O<sup>6</sup>-position of guanine induced by alkylating agents. *MGMT* promoter hypermethylation and reduced expression has been found in some primary human carcinomas. We studied DNA methylation of CpG islands of the *MGMT* gene and its relation with *MGMT* protein expression in human epithelial ovarian carcinoma.

**Methods:** A total of 88 epithelial ovarian cancer (EOC) tissue samples, 14 low malignant potential (LMP) tumours and 20 benign ovarian tissue samples were analysed for *MGMT* promoter methylation by nested methylation-specific polymerase chain reaction (MSP) after bisulphite modification of DNA. A subset of 64 EOC samples, 10 LMP and benign tumours and five normal ovarian tissue samples were analysed for protein expression by immunohistochemistry.

**Results:** The methylation frequencies of the *MGMT* gene promoter were found to be 29.5, 28.6 and 20 per cent for EOC samples, LMP tumours and benign cases, respectively. Positive protein expression was observed in 93.8 per cent of EOC and 100 per cent in LMP, benign tumours and normal ovarian tissue samples. Promoter hypermethylation with loss of protein expression was seen only in one case of EOC.

**Interpretation & conclusions:** Our results suggest that *MGMT* promoter hypermethylation does not always reflect gene expression.

**Key words** Epithelial ovarian tumour - *MGMT* - ovarian cancer - promoter methylation - protein expression

DNA methylation, the addition of a methyl group to the carbon-5 position of cytosine residues, is the common covalent modification of human DNA and occurs almost exclusively at cytosines that are followed immediately by a guanine (so-called CpG dinucleotides). The bulk of the genome displays a

clear depletion of CpG dinucleotides and those that are present are nearly always methylated. In contrast, small stretches of DNA, known as CpG islands, are comparatively rich in CpG dinucleotides and are nearly free of methylation. These CpG islands are usually located within the promoter regions of human

gene and methylation within the islands has shown to be associated with transcriptional inactivation of the corresponding gene. Alterations in DNA methylation might be pivotal in the development of cancer. The pattern of DNA methylation observed in cancer generally shows a dramatic shift compared with that of normal tissue. Such changes in methylation have a central role in tumorigenesis; in particular, methylation of CpG islands has been shown to be important in transcriptional repression of numerous genes that function to prevent tumour growth or development. Studies of DNA methylation in cancer have thus opened up new opportunities for diagnosis, prognosis and ultimately treatment of human tumours<sup>1</sup>.

The O<sup>6</sup>-alkyl guanine-DNA alkyl transferase (AGT) also known as O<sup>6</sup>-methylguanine DNA methyltransferase (*MGMT*), is the DNA repair protein responsible for removing alkylation adducts from the O<sup>6</sup>-position of guanine in DNA<sup>2,3</sup>. The promoter CpG island hypermethylation associated gene silencing of *MGMT* is associated with a wide spectrum of human cancers such as oesophagus, lung, colon and cervix<sup>4-7</sup>. However, the functional role of CpG island methylation in *MGMT* silencing is still controversial in ovarian cancer.

Ovarian cancer is the leading cause of death among women with gynaecologic cancers<sup>8</sup>. Despite advances in cancer research and treatment, survival for patients with ovarian cancer remains low; >50 per cent of patients die within five years of their ovarian cancer diagnosis<sup>9</sup>. This poor survival rate is due in part to the lack of sensitive and specific methods of early detection. Because symptoms in early-stage ovarian cancer generally are non-specific, patients with ovarian cancer usually are diagnosed with either stage III or stage IV disease that has already spread beyond the ovary<sup>10</sup>. A better understanding of the molecular mechanisms that are responsible for ovarian cancer development and progression will help improve the diagnosis and treatment of the disease.

The aim of this investigation was to study *MGMT* CpG island hypermethylation and to investigate whether *MGMT* CpG island hypermethylation reflects the expression of the gene in human epithelial ovarian carcinoma in the Indian population.

### Material & Methods

*Sample collection and DNA extraction*: A total of 88 primary epithelial ovarian tumour samples, 14 low malignant potential (LMP) tumour samples and 20

benign ovarian tissue samples from patients with ovarian cancer who had not undergone any prior treatment were obtained from the Department of Gynaec Oncology, Kidwai Memorial Institute of Oncology, Bangalore, India, from August 2010 to September 2012. Metastatic carcinoma of the ovary and tumours of germ cell and stromal cell were excluded from the study. Fifteen normal ovarian tissues from women without a family history of ovarian and breast cancer were also collected at the time of oophorectomy. The study was approved by the Institutional Review Board and Medical Ethics Committee and written informed consent was taken from the patients enrolled into the study. Tissue specimens were obtained at the time of surgery and stored at -70°C until DNA extraction. Tumour rich (80-90%) areas which were non-necrotic with very little stroma were selected. Histological typing was carried out according to WHO standards and staging of tumours assigned according to the International Federation of Gynecology and Obstetrics (FIGO) system<sup>11</sup>. The clinicopathological variables included in the study were FIGO stage, histological grade and subtype, mensus status, presence or absence of ascites and pre-operative CA-125 levels. 57 (65%) were grade 3 tumours and 64 (73%) cases had clinical stage III disease. Serous tumours were the most common histological subtype (52[59%]).

Genomic DNA was extracted from 25 mg of tissue using QIAamp DNA Mini Kit (Qiagen, USA) following the manufacturer's instructions. DNA concentration was quantified spectrophotometrically using the Nanodrop spectrophotometer (Thermo Fischer Scientific Inc., USA) and the extracted DNA was stored at -20°C until processed.

*Methylation specific polymerase chain reaction (MSP)*: DNA methylation pattern in CpG island of *MGMT* gene was determined by nested MSP of bisulphite converted DNA. DNA bisulphite conversion was performed using a commercially available kit (EZ DNA methylation kit, Zymo Research, California, USA) following the manufacturer's instructions. DNA was treated with sodium bisulphite, to convert all unmethylated cytosine to uracil, whereas methylated cytosine remained unchanged. Bisulphite converted DNA was stored at -70°C until used.

For the first round of PCR amplification, 2 µl of bisulphite modified DNA was taken in a final volume of 50 µl reaction mixture containing template (~100 ng), 1.5 mM/l MgCl<sub>2</sub>, 10 pM/l of each forward and reverse primer (Sigma Aldrich, USA), 0.2 mM/l of each of the

four dNTPs, 5µl of 10x PCR buffer (NEB, England) and 1U of Taq polymerase (NEB, England). Amplification was carried out in a S1000 Thermal Cycler (Bio-Rad Laboratories, USA) under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles each of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 30 sec followed by a final extension at 72°C for 4 min. The resulting PCR products of *MGMT* 135bp, served as a template for the second MSP. The PCR product from the first step was diluted 10-folds and subjected to the second round of PCR with primers specific for methylated and unmethylated sequences of *MGMT* promoter.

The primer sequences used for nested MSP, methylated and unmethylated PCR are given in Table I. Amplification was carried out in a S1000 Thermal Cycler under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles each of denaturation at 95°C for 15 sec, annealing at 62°C for 15 sec and extension at 72°C for 15 sec followed by a final extension at 72°C for 7 min. The resulting PCR products for *MGMT* were 81 and 93bp for methylated and unmethylated sequences, respectively (Fig. 1).

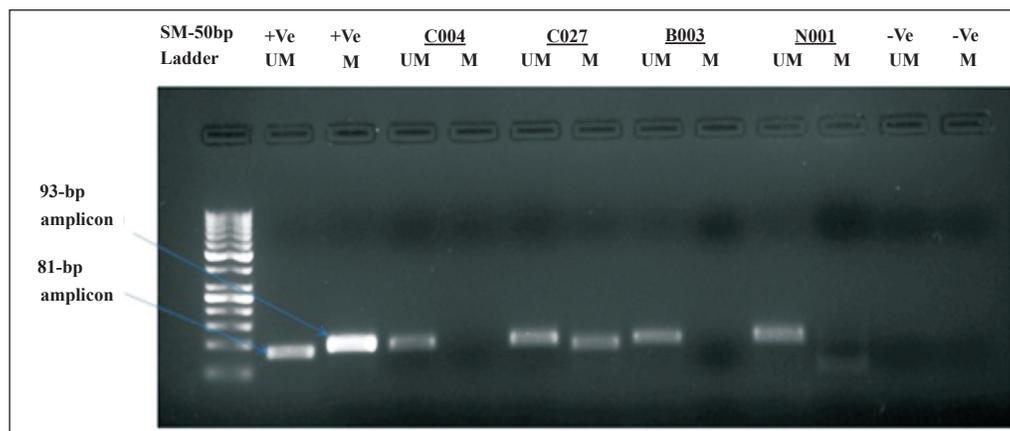
CpGenome Universal Methylated DNA (Zymo Research Corp, USA) was used as a positive control for amplification of methylated alleles. Peripheral blood lymphocyte DNA was used as unmethylated control. Water blank without added DNA template was included as negative PCR control in each assay. The PCR products were subjected to electrophoresis in 3 per cent agarose gel stained with ethidium bromide and visualized using a UV illuminator. A 50 bp ladder (Fermentas, Germany) was used as molecular weight standard.

**Immunohistochemistry:** Immunohistochemistry (IHC) staining of the *MGMT* proteins products was done on a subset of the study cases (64 epithelial ovarian tumours, 10 LMP and benign tumours and 5 normal ovarian tissues).

**Construction of tissue microarray (TMA):** The formalin fixed paraffin-embedded tissues were used for constructing TMA blocks. Selected cancer foci were marked on hematoxylin and eosin (H&E)-stained sections. A tissue-arranging instrument was used to acquire cylindrical tissue cores with a diameter of 2 mm from histologically representative areas of the

**Table I.** Primer sequences of nested methylation-specific polymerase chain reaction

MSP reaction	Sense primer	Antisense primer
Nested	5' GAGTTTGGGATATGTTGGGATAGTT 3'	5' AAACCTCCTCACTCTTCCCAAAAAC 3'
Unmethylated	5' TTTGTGTTTTGATGTTTGTAGGTTTTTGT 3'	5' AACTCCACACTCTTCCAAAAACAAAACA 3'
Methylated	5' TTTCGACGTCGTAGGTTTTTCGC 3'	5' GCACTCTTCCGAAAACGAAACG 3'



**Fig. 1.** Methylation analysis of *MGMT* gene. Agarose gel showing representative product of MSP analysis of *MGMT* gene in epithelial ovarian tumours. In each case, CpGenome universal methylated genomic DNA was used as a positive (+ve) control for methylated alleles and peripheral blood mononuclear cell (PBMC) DNA from normal healthy subjects as positive control for unmethylated alleles. PCR products in lane UM indicate the presence of an unmethylated allele, whereas PCR products in lane M indicate the presence of a methylated allele. C004, C027 are carcinomas, B003 is benign adenoma and N001 is a normal tissue. No template control was used as a negative control.

donor blocks. Thirty six tissue cores were composited into a single recipient paraffin block at defined array positions. Two tissue cores were obtained from each specimen and represented in duplicate on the array. Five  $\mu\text{m}$  sections were cut from the TMA block and mounted on 2 per cent aminopropyl triethoxysilane (Sigma, USA) coated glass slides. The presence of tumour tissue on the arrayed samples was verified on H&E section to confirm tissue morphology.

**Immunostaining:** Five  $\mu\text{m}$  thick sections were cut from paraffin-embedded blocks of TMA, dewaxed in xylene, and rehydrated in a graded series of alcohol washes down to water. Steam antigen retrieval was performed for 20 min in Tris-EDTA, pH 9.0. The sections were treated with 3 per cent  $\text{H}_2\text{O}_2$  for 20 min to block any endogenous peroxidase activity. Non-specific binding sites were blocked with 2 per cent skimmed milk for 30 min. The sections were incubated with primary mouse anti-human MGMT monoclonal antibody (1:50 dilution; MAB16200, clone MT3.1, Chemicon International Inc, Temecula, CA, USA) for one hour 30 min at room temperature, then with secondary and tertiary goat anti-mouse antibody (Biogenex, Bangalore, India) for 30 min each at room temperature. Colour development was performed using diaminobenzidine-hydrogen peroxide for 10 min. The sections were then counterstained with hematoxylin. Glioma tissue section stained with MGMT antibody was used as positive control, whereas section with no primary antibody was used as negative control to rule out non-specific reaction. MGMT expression of malignant cells was interpreted as negative when less than 15 per cent of cells showed nuclear staining and positive when more than 15 per cent of cells stained positive for MGMT<sup>12</sup>. Only nuclear staining was considered for evaluation.

**Statistical analysis:** Methylation frequencies between patients and controls were analysed using Fisher's exact probability test (where sample numbers were less than 5) or  $\chi^2$  (where sample number exceeded 5).

All statistical analyses were performed with SPSS 21.0 version statistical software (IBM, India).

## Results

The age of the patients ranged from 23 to 72 yr and the median age was 48 yr. All tumours were of epithelial origin and serous tumours were the most common histological type [52 (59%)]. Most of the tumours were grade III [57 (65%)] and advanced stage [64 (73%)].

**MGMT promoter methylation status by MSP:** Methylation frequencies determined by MSP are shown in Table II. The methylation rate of MGMT promoter in epithelial ovarian carcinoma tissues was 29.5 per cent in epithelial ovarian tumour samples, 28.6 per cent in low malignant potential tumours and 20 per cent in benign tumours. No methylation was observed in normal ovarian tissue samples. A significant difference in methylation frequencies was found between the normal ovarian tissue group and the malignant and borderline tumour groups ( $P < 0.05$ ).

**Relation of MGMT methylation with clinico-pathological parameters:** Table III presents the relation between the methylation status of MGMT and clinical and pathological features. The methylation frequencies significantly differed among tumour subtypes ( $P < 0.05$ ). Endometrioid adenocarcinomas did not show any methylation. While clear cell adenocarcinomas showed 60 per cent methylation.

**Association of MGMT promoter methylation and protein expression:** Representative examples of IHC staining results are shown in Fig. 2 A-E. The tumours were categorized into two groups based on IHC results as positive expression (>15% staining) or negative expression (<15% staining). Abundant nuclear MGMT expression was seen in 60 of 64 (93.8%) epithelial ovarian carcinomas (EOC). All LMP and benign tumours and normal ovaries showed positive protein expression (Table IV).

**Table II.** Methylation frequencies of study subjects

Genes	Tumour type			
	Epithelial ovarian tumour (88)	Low malignant potential tumour (14)	Benign tumour (20)	Normal (15)
MGMT (U)	62 (70.5)	10 (71.4)	16 (80)	15 (100)
MGMT (M)	26 (29.5)*	4 (28.6)*	4 (20)	0 (0)

U, unmethylated; M, methylated. Values in parentheses are percentages.  
\* $P < 0.05$  compared to normal ovarian tissue

**Table III.** Correlation of MGMT methylation with clinico-pathological parameters

Clinical and pathological parameters	MGMT methylated cases N (%)
<b>Ovarian tumours (88)</b>	<b>26 (29.5)</b>
FIGO stage	
I (14)	2 (14.3)
II (8)	3 (37.5)
III (64)	21 (32.8)
IV (2)	0 (0)
Type of tumour*	
Serous adenocarcinoma (52)	11 (21.2)
Mucinous adenocarcinoma (9)	5 (55.6)
Endometrioid adenocarcinoma (5)	0 (0)
Clear-cell adenocarcinoma (5)	3 (60)
Poorly differentiated adenocarcinoma (17)	7 (41.2)
Histological grade	
G1 (10)	3 (30)
G2 (15)	3 (20)
G3 (57)	18 (31.6)
Undetermined (6)	2 (33.3)
Menopausal status	
Post menopause (57)	15 (26.3)
Pre menopause ( 31)	11 (35.5)
Presence of ascites	
Present (67)	19 (28.4)
Absent (21)	7 (33.3)
Pre-operative CA125 values (U/ml)	
0-35 (5)	2 (40)
35-110 (5)	2 (40)
110-1000 (5)	17 (34.7)
>1000 (5)	5 (17.2)
Borderline tumours (14)	
Serous borderline (7)	2 (28.6)
Mucinous borderline (7)	2 (28.6)
Benign tumours (20)	
Serous cystadenoma (13)	3 (23.1)
Mucinous cystadenoma (7)	1 (14.3)
Normal ovaries (15)	0 (0)

\*P<0.05  
FIGO, International Federation of Gynecology & Obstetrics;  
CA 125, cancer antigen 125

Table V summarises the association between protein expression with methylation of *MGMT*. Protein expression levels of *MGMT* were not significantly associated with promoter hypermethylation of *MGMT* ( $P=1.000$ ) and promoter hypermethylation with loss of protein expression was seen only in one case of EOC.

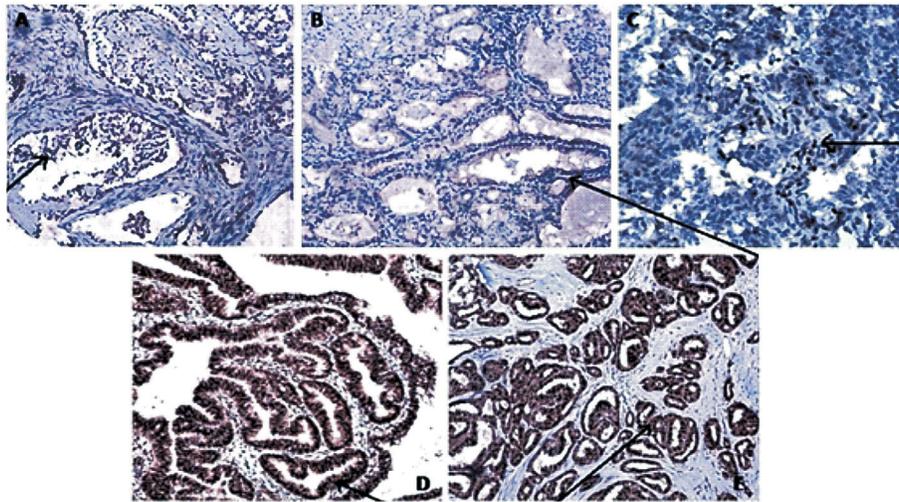
### Discussion

DNA methylation is an inheritable epigenetic change in human cancers and the transcriptional silencing by hypermethylation of CpG islands in the promoter region is being recognised as a common mechanism for the inactivation of various tumour suppressor genes and also affects a number of molecular pathways in human cancer<sup>13</sup>. The cellular DNA repair protein MGMT functions as a DNA repair enzyme that removes the mutagenic alkyl adducts from the O<sup>6</sup> – position of guanine. Tumours appear to be heterogenous with respect to *MGMT* expression and in a subset of cancer cells its expression is silenced due to abnormal promoter methylation<sup>14</sup>.

Our results demonstrated that the methylation observed in the *MGMT* promoter in epithelial ovarian carcinoma samples did not relate with *MGMT* expression as shown by immunohistochemistry, a finding that was unexpected given that methylation is associated with absence of MGMT protein product in gliomas and other cancers<sup>15</sup>.

In the analysed epithelial ovarian carcinoma samples, 29.5 per cent had a detectable methylated *MGMT* promoter, while the promoter hypermethylation was 28.6 per cent and 20 per cent for LMP and the benign tumours, respectively. No methylation was observed in normal ovaries. Jiase *et al*<sup>16</sup> observed that *MGMT* mRNA expression was lower in ovarian tumours than in normal ovaries, with 31.1 per cent of the promoters methylated in malignant ovarian carcinomas<sup>15</sup>. Another study has reported a high frequency (48%) of methylation of *MGMT* promoter<sup>17</sup>. A study using ovarian cancer cell lines has reported 23 per cent hypermethylation of *MGMT*<sup>18</sup>, while another study of ovarian granulosa cell tumours has reported 33 per cent hypermethylation<sup>8</sup>.

DNA methylation-dependent silencing of gene expression in cancer results in loss of protein expression and consequently protein function. The relationship between methylation and expression was analysed for EOC cases, LMP tumours, benign cases and 5 normal ovaries. Loss of protein was noted in only 4 of 64 EOC cases. Hypermethylation in *MGMT* gene and



**Fig. 2.** Immunohistochemistry (IHC) staining for *MGMT* at 10x magnification. **A.** *MGMT* positive (arrow). **B.** *MGMT* negative (arrow). **C.** Ovarian cancer case methylated at *MGMT* promoter showing loss of *MGMT* protein expression (arrow). **D. & E.** Two ovarian cancer cases unmethylated at *MGMT* promoter showing different levels of protein expression (arrow).

**Table IV.** Tissue expression of epithelial ovarian carcinoma (EOC), low malignant potential (LMP) tumours, benign tumours and normal ovaries

No. of cases	Positive expression N (%)	Negative expression N (%)
EOC (64)	60 (93.8)	4 (6.2)
LMP (10)	10 (100)	0 (0)
Benign tumours (10)	10 (100)	0 (0)
Normal ovaries (5)	5 (100)	0 (0)

**Table V.** Association of *MGMT* promoter methylation and protein expression

Gene	Tumour type	Methylation status	Expression	
			Positive N (%)	Negative N (%)
<i>MGMT</i>	Epithelial ovarian cancer (64)	U (46)	43 (93.5)	3 (6.5)
		M (18)	17 (94.4)	1 (5.6)
	Low malignant potential tumours (10)	U (7)	7 (100)	0
		M (3)	3 (100)	0
	Benign tumours (10)	U (7)	7 (100)	0
		M (3)	3 (100)	0
	Normal (5)	U (5)	5 (100)	0

its reduced expression was seen only in one case of carcinoma.

Rodriguez *et al*<sup>19</sup> showed poor correlation between *MGMT* promoter methylation and *MGMT* expression by immunohistochemistry in glioblastoma specimens. Rimel and colleagues<sup>20</sup> studied *MGMT* promoter methylation and protein expression in 21

primary ovarian tumours and found methylation in only one case of endometrioid cancer which also had high level of *MGMT* expression showing no correlation between promoter methylation and protein expression. Similar results have been reported by others<sup>4,13,20,21</sup>. Loss of *MGMT* expression was detected in 14.0 per cent ovarian epithelial cancers. In 34 cases where MSP results were available, *MGMT* promoter

hypermethylation was detected in 14.7 per cent cases with mucinous or clear cell carcinomas, but not in any of the other histologic types<sup>21,22</sup>.

Our results showed a significant promoter methylation of the DNA repair gene *MGMT* in epithelial ovarian carcinoma. Our data also showed that methylation in the promoter region of *MGMT* was not related with the expression of *MGMT* gene suggesting that the relationship between methylation and expression was not absolute. It is possible that *MGMT* promoter DNA methylation plays only an indirect role in the regulation of *MGMT* expression<sup>23</sup>. It has been suggested that DNA methylation in cancer could be a secondary process to an initial dramatic change in expression<sup>24,25</sup>. Mechanisms such as gene deletion or mutation have been implicated as alternative mechanisms of gene silencing and partial methylation might account for protein expression in spite of evidence of methylated *MGMT* promoter<sup>26</sup>.

In conclusion, our results indicate that it is not always true for all of the tumour suppressor genes or DNA repair genes to show a positive association between promoter hypermethylation and loss of expression in cancers.

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### References

1. Strathdee G, Brown R. Aberrant DNA methylation in cancer: potential clinical interventions. *Expert Rev Mol Med* 2002; 4 : 1-17.
2. Ludlum DB. DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal. *Mutat Res* 1990; 233 : 117-26.
3. Pegg AE. Repair of O<sup>6</sup>-alkylguanine by alkyltransferases. *Mutat Res* 2000; 462 : 83-100.
4. Ogino S, Meyerhardt JA, Kawasaki T, Clark JW, Ryan DP, Kulke MH, *et al.* CpG island methylation, response to combination chemotherapy, and patient survival in advanced microsatellite stable colorectal carcinoma. *Virchows Arch* 2007; 450 : 529-37.
5. Rodriguez MJ, Acha A, Ruesga MT, Rodriguez C, Rivera JM, Aguirre JM. Loss of expression of DNA repair enzyme *MGMT* in oral leukoplakia and early oral squamous cell carcinoma. A prognostic tool? *Cancer Lett* 2007, 245 : 263-8.
6. Ishii T, Murakami J, Notohara K, Cullings HM, Sasamoto H, Kambara T, *et al.* Oesophageal squamous cell carcinoma may develop within a background of accumulating DNA methylation in normal and dysplastic mucosa. *Gut* 2007; 56 : 13-9.
7. Weaver KD, Grossman SA, Herman JG. Methylated tumor-specific DNA as a plasma biomarker in patients with glioma. *Cancer Invest* 2006; 24 : 35-40.
8. Ozols RF, Schwartz PE, Eifel PJ. Ovarian cancer, peritoneal carcinoma, and fallopian tube carcinoma. In: DeVita VT Jr., Hellman S, Rosenberg SA, editors. *Cancer: principles and practice of oncology*, Section 32.4, 7<sup>th</sup> ed. Philadelphia: Lippincott, Williams and Wilkins; 2005. p. 1364-98.
9. Colombo N, Van Gorp T, Parma G, Amant F, Gatta G, Sessa C, *et al.* Ovarian cancer. *Crit Rev Oncol Hematol* 2006; 60 : 159-79.
10. Goff BA, Mandel LS, Melancon CH, Muntz HG. Frequency of symptoms of ovarian cancer in women presenting to primary care clinics. *JAMA* 2004; 291 : 2705-12.
11. Prat J; FIGO Committee on Gynecologic Oncology. Staging classification for cancer of the ovary, fallopian tube and peritoneum. *Int J Gynecol Obstet* 2014; 124 : 1-5.
12. Shah N, Lin B, Sibenaller Z, Ryken T, Lee H, Yoon J-G, *et al.* Comprehensive analysis of *MGMT* promoter methylation: Correlation with *MGMT* expression and clinical response in GBM. *PLoS One* 2011; 6 : e16146.
13. Dhillon VS, Young AR, Husain SA, Aslam M. Promoter hypermethylation of *MGMT*, *CDH1*, *RAR-β* and *SYK* tumor suppressor genes in granulose cell tumors (GCTs) of ovarian origin. *Br J Cancer* 2004; 90 : 874-81.
14. Cankovic M, Mikkelsen T, Rosenblum ML, Zarbo RJ. A simplified laboratory validated assay for *MGMT* promoter hypermethylation analysis of glioma specimens from formalin-fixed paraffin-embedded tissue. *Lab Invest* 2007; 87 : 392-7.
15. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O<sup>6</sup>-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res* 1999; 59 : 793-7.
16. Jiaye A, Qingyi W, Zhensheng L, Karen H L, Xi C, Gordon BM, *et al.* Messenger RNA expression and methylation of candidate tumor-suppressor genes and risk of ovarian cancer—a case-control analysis. *Int J Mol Epidemiol Genet* 2010; 1 : 1-10.
17. Furlan D, Carnevali I, Marcomini B, Cerutti R, Dainese E, Capella C, *et al.* The high frequency of de novo promoter methylation in synchronous primary endometrial and ovarian carcinomas. *Clin Cancer Res* 2006; 12 : 3329-36.
18. Imura M, Yamahita S, Cai LY, Furuta J, Wakabayashi M, Yasugi T, *et al.* Methylation and expression analysis of 15 genes and three normally-methylated genes in 13 ovarian cancer cell lines. *Cancer Lett* 2006; 241 : 213-20.
19. Rodriguez FJ, Thibodeau SN, Jenkins RB, Schowatler KV, Caron BL, O'neill BP, *et al.* *MGMT* immunohistochemical expression and promoter methylation in human glioblastoma. *Appl Immunohistochem Mol Morphol* 2007; 16 : 59-65.
20. Rimel BJ, Huettner P, Powell MA, Mutch DG, Paul J. Absence of *MGMT* promoter methylation in endometrial cancer. *Gynecol Oncol* 2009, 112 : 224-8.

21. Brell M, Tor tosa A, Verger E, Gil GM, Vinolas N, Villa S, *et al.* Prognostic significance of O<sup>6</sup>-methylguanine-DNA methyltransferase determined by promoter hypermethylation and immunohistochemical expression in anaplastic gliomas. *Clin Cancer Res* 2005; *11* : 5167-74.
22. Roh HJ, Suh DS, Choi KU, Yoo HJ, Joo WD, Yoon MS. Inactivation of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) by promoter hypermethylation: A key factor of epithelial ovarian carcinogenesis in specific histologic types. *J Obstet Gynaecol Res* 2011; *37* : 851-60.
23. Pieper RO, Patel S, Ting SA, Futscher BW, Costello JF. Methylation of CpG island transcription factor binding sites is unnecessary for aberrant silencing of the human *MGMT* gene. *J Biol Chem* 1996; *271* : 13916-24.
24. Turker MS. Gene silencing in mammalian cells and the spread of DNA methylation. *Oncogene* 2002; *21* : 5388-93.
25. Clark SJ, Melki J. DNA methylation and gene silencing in cancer: which is the guilty party? *Oncogene* 2002; *21* : 5380-7.
26. Thomas M, Christoph B, Osman El-Maarri, Anika H, Denise E, Johannes S, *et al.* Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *J Mol Diagn* 2007; *9* : 368-81.

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