Promoter hypermethylation of MGMT, CDH1, RAR-β and SYK tumour suppressor genes in granulosa cell tumours (GCTs) of ovarian origin

VS Dhillon*1, AR Young1, SA Husain2 and M Aslam2

1Department of Molecular and Clinical Genetics, Royal Prince Alfred Hospital, Camperdown NSW 2050, Australia; 2Cytogenetics Laboratory, Department of Biosciences, Jamia Millia Islamia, New Delhi 110 025, India

Ovarian carcinoma (OC) is a leading cause of death among women throughout the world. A number of cancer-associated genes have been shown to be inactivated by hypermethylation of CpG islands during tumorigenesis. We tested the hypothesis that methylation status of MGMT, CDH1, RAR-β and SYK could be important in the ovarian tumorigenic process and can lead to the gene(s) inactivation. Therefore, we assessed the promoter hypermethylation of MGMT, CDH1, RAR-β and SYK in 43 ovarian granulosa cell tumours (GCTs) (adult type) using methylation-specific PCR. These tumours are relatively rare, accounting for approximately 3% of all ovarian cancers. Hypermethylation of MGMT (in 14 tumours), CDH1 (in nine tumours), RAR-β (in eight tumours) and SYK (in seven tumours) have been found. Selective loss of RAR-β and RAR-β2 mRNA has been found in seven patients, while that of MGMT and SYK in three patients who also show aberrant methylation in promoter region of RAR-β in addition to MGMT, SYK and CDH1 genes. Promoter CpG hypermethylation may be an alternative to mutation(s) to inactivate tumour suppressor genes such as MGMT, CDH1, RAR-β and SYK, and this can also be an early event in the pathogenesis of OCs. Moreover, hypermethylation of the MGMT and CDH1, MGMT and RAR-β and CDH1 and RAR-β promoters occurred concordantly (P = 0.001, 0.0421 and 0.0005 respectively; Fischer’s exact test). In addition to this, monosomy 22 and trisomy 14 have also been found in 10 tumours. It is clear from the results that hypermethylation of the promoter region of these tumour suppressor genes, monosomy 22 and trisomy 14, may be critical steps in the tumorigenesis, which consequently play a permissive role for tumour aggressiveness. All these events might play an important role in the early clinical diagnosis of the disease. Our results, therefore, suggest a potential role for epigenetic modification of these critical tumour suppressor genes in pathways relevant to the transformation and differentiation of rare type of ovarian cancer (GCTs).

British Journal of Cancer (2004) 90, 874–881. doi:10.1038/sj.bjc.6601567 www.bjcancer.com

Keywords: ovarian carcinoma; hypermethylation of MGMT; CDH1; RAR-β and SYK gene

Ovarian tumours are relatively common, and are the most lethal tumour of the female genital tract (Evan et al, 1999). Among ovarian cancers, granulosa cell tumours (GCTs) are relatively rare, accounting for approximately 3% of all ovarian cancers. The aetiology of these GCTs remains unclear. It is now accepted that accumulation of multiple genetic aberrations can lead to the development of most malignancies. DNA replication errors (RER) have been detected in epithelial ovarian cancer, as well as in other human cancer types, and a DNA mismatch repair deficiency may be involved in their development and or progression (Suzuki et al, 2000). Genetic or epigenetic alterations in a variety of genes are fundamental to the processes of growth, cell proliferation, differentiation and programmed cell death and removal. Each alteration may be mediated through gross chromosomal changes and, hence, has the potential to be detected cytogenetically (Sandberg, 1991). It is generally thought that the events causing the activation of certain oncogenes and/or inactivation of tumour suppressor genes lead to tumour development and progression, which is caused by genetic alterations such as chromosome deletions and or loss of function mutations in the coding regions (in CpG sites) of the tumour suppressor genes.

O6-methylguanine-DNA methyltransferase (MGMT; 10q26; GenBank Accession No. U95038) is a DNA repair gene, which removes methyl groups as well as larger adducts at O6 of guanine. The alkylation of DNA at O6 position of guanine is associated with the formation of DNA mutations in cancers. CpG islands within −249 to + 259 relative to transcriptional start site were chosen to study the role of promoter methylation on gene silencing. Methylation of MGMT gene has been reported in various carcinomas such as gliomas, non-small-cell lung carcinoma, lymphoma, head and neck carcinoma and colorectal cancers (Esteller et al, 1999b, 2000; Rosas et al, 2001). E-cadherin is an M, 120 000 transmembrane glycoprotein (16q22.1; GenBank Accession No. D49685) expressed on the surface of epithelial cells and is essential for the
Promoter hypermethylation in ovarian carcinoma
VS Dhillion et al

The subjects were 43 patients affected with GCTs of ovaries without a positive family history. All of them were untreated at the time of the study. The tumour grading is as follows: 19 FIGO stage IA, 10 FIGO stage IB and 14 FIGO stage IC. All these diagnoses were reviewed by a gynaecologic pathologist, and the tumours were assessed using standard criteria (Russell and Bannatyne, 1989). An informed consent was taken from all the subjects prior to the study. This study was approved by the Health Research Ethics Board of the Faculty of Natural Sciences. In all, 25 G-band metaphases were obtained after a 5-day-old culture of an overnight collagenase disaggregated specimen of the tumour.

Methylation-Specific PCR (MSP)
DNA methylation patterns in CpG islands of tumour suppressor genes MGMT, CDH1, RAR-β and SYK were determined by chemical modification with sodium bisulphite. Briefly, 1 μg DNA was denatured by NaOH (50 μl, final concentration, 0.2 M) for 10 min at 37 °C; 1 μg of salmon sperm DNA (Sigma) was added as carrier before modification. Freshly prepared 30 μl of hydroquinone (10 mM, Sigma) and 520 μl of sodium bisulphite (3 M, pH 5.0, Sigma) were mixed and the samples were incubated under mineral oil at 55 °C for 16 h. The DNA samples were desalted through Wizard columns (Promega, Madison, WI, USA), then desulphonated by NaOH (final concentration, 0.3 M) for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in water and used immediately or stored at −20°C. PCR primers that distinguish between these methylated and unmethylated DNA sequences were used. Primer sequences of all genes for both the methylated and the unmethylated form, unmethylated DNA sequences were used. Primer sequences of all genes were determined by MethylGreen PCR (Applied Biosystems, NJ, USA). Amplification was carried out in a 9700 Perkin Elmer thermal cycler under the following conditions: 95°C for 10 min; 35 cycles of 95°C for 45 s, the specific

Table 1

| S. no. | Name and type | Primer sequence | PCR conditions | Cycles | bp |
|-------|--------------|-----------------|----------------|--------|----|
| 1     | MGMT-Met: F  | 5'-TTT CGA CGT TCG TAG GTT TCT GC-3' | 95°C x 30s; 61°C x 45s; 72°C x 45 s | 35 | 81 |
|       | R            | 5'-GCA CTC CGA AAA CGA AAC G-3' | 95°C x 30s; 61°C x 45s; 72°C x 45 s | 35 | 93 |
| 2     | MGMT-UNM: F  | 5'-TTT GTG TTT TGA TGT TAG GTT TTT GT-3' | 95°C x 30s; 61°C x 45s; 72°C x 45 s | 40 | 112 |
|       | R            | 5'-AAC TCC ACA TTC TCT CAA CCA AAC A-3' | 95°C x 30s; 61°C x 45s; 72°C x 45 s | 40 | 120 |
| 3     | CDH1-Met: F  | 5'-TGT AGT AGT GTA TTT ATT ATT AGT GGC GTC-3' | 95°C x 30s; 64°C x 30s; 72°C x 30 s | 35 | 235 |
|       | R            | 5'-CCA ATG CCG GGG GGG GGG ATT ATT-3' | 95°C x 30s; 64°C x 30s; 72°C x 30 s | 35 | 233 |
| 4     | CDH1-UNM: F  | 5'-TTG TTG TAG TTA TGT ATT ATT TAT TGT TTG CGT C-3' | 95°C x 30s; 62°C x 30s; 72°C x 30 s | 35 | 243 |
|       | R            | 5'-ACA CCA AAT ACA ATG TTA ACA CAA A-3' | 95°C x 30s; 62°C x 30s; 72°C x 30 s | 35 | 243 |
| 5     | RARβ-Met: F  | 5'-GGA GTG TAG TAG TTC GCG TAG GGT TTA TC-3' | 95°C x 30s; 58°C x 30s; 72°C x 30 s | 35 | 235 |
|       | R            | 5'-CCG AAT CCT CCC ACC ACG-3' | 95°C x 30s; 58°C x 30s; 72°C x 30 s | 35 | 233 |
| 6     | RARβ-UNM: F  | 5'-TTA GTA GTG TGG GTA GGG ATT ATT-3' | 95°C x 30s; 67°C x 30s; 72°C x 30 s | 35 | 140 |
|       | R            | 5'-CCA AAT CCT CCC ACC ACA-3' | 95°C x 30s; 67°C x 30s; 72°C x 30 s | 35 | 140 |
| 7     | SYK-Met: F   | 5'-CCA TTT GCG GGG TGT TTT CC-3' | 95°C x 30s; 67°C x 30s; 72°C x 30 s | 35 | 140 |
|       | R            | 5'-ATA TTT GGT TGG TTT GTG TGG-3' | 95°C x 30s; 67°C x 30s; 72°C x 30 s | 35 | 140 |
| 8     | SYK-UNM: F   | 5'-ACT TTT AAT ACC TCA CAC CCA AAC-3' | 95°C x 30s; 67°C x 30s; 72°C x 30 s | 35 | 140 |

© 2004 Cancer Research UK
British Journal of Cancer (2004) 90(4), 874 – 881
Genetics and Genomics
annealing temperature for each gene for 1 min, and 72°C for 60 s; followed by a final 10 min extension at 72°C. Following sequencing, the primer pair was used to amplify bisulphite-modified DNA-containing gene for E-cadherin: (forward) 5'-GTG TAG TTT TGG GTA GGG GTT-3' and (reverse) 5'-ACT ACT CCT CAA AAA ACC CAT AAC TAA-3'. The PCR cycle consisted of initial denaturation at 95°C for 5 min, 30 cycles (95°C × 30 s, 50°C × 30 s and 72°C × 30 s) followed by a final extension at 72°C for 5 min. The PCR product was then diluted 1:50 with sterile distilled water and 2 µl of this 1:50 diluted product was then used for nested PCR using methylated and unmethylated primer pairs. Similarly, the following sequencing primer pair was used to amplify bisulphite-modified DNA-containing gene for SYK: (forward) 5'-GAT TAA GAT ATA TTT TAG GGA ATA TG-3' and (reverse) 5'-CAC CTA TAT TTT ATT CAC ATA ATT TC-3'. The PCR cycle consisted of hot start at 95°C × 5 min and 35 cycles (95°C × 45 s, 58°C × 50 s and 72°C × 45 s) followed by a final extension at 72°C for 5 min. The PCR product was then diluted 1:50 with sterile distilled water and 2 µl of this 1:50 diluted product was then used for nested PCR using methylated and unmethylated primer pairs. Each PCR product (10 µl) was directly loaded onto 6% non-denaturing polyacrylamide gels, stained with ethidium bromide and directly visualised under UV illumination. The gel for all samples was repeated twice to confirm the methylation status.

Statistical differences were assessed with Fisher's exact test by InStat 3 Windows software. Two-sided test were used to determine the significance. P-values less than 0.05 were regarded as statistically significant.

RT-PCR

RAR-β and RAR-β-2

Total RNA was prepared using RNeasy mini-kit (Qiagen, CA, USA) as per the manufacturer’s recommendations. In brief, total RNA was extracted from the specific cancerous tissues. Cell lysates were then homogenised by passing repeatedly through a 23-gauge needle followed by spinning through a Qiashredder (Qiagen, CA, USA). RNA concentrations were determined by measuring the absorbance (260 nm UV) using a spectrophotometer (Pharmacia). The total RNA (2 µg) was used for the generation of cDNAs using Superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD, USA). The following primers were used to detect RAR-β and RAR-β2 transcripts: RAR-β forward 5'-ACC AGG TCT GAG GAA CTC and RAR-β reverse 5'-AGG CGG CCT TCA GCA GGG TAA TTT-3', and for RAR-β2 forward (in exon 3), 5'-GCA TGG CAG AGT GCC CTA TC-3'; reverse (in exon 6), 5'-TCC CAG AGT CAT CCC TGC TTC AT-3'. PCR amplification was performed for 30 cycles at 95°C for 30 s, 60°C for 30 s (62°C for RAR-β2) and 72°C for 45 s (60 s for RAR-β2). Human GAPDH was used as an internal control.

MGMT gene

The following primer sequences were used for MGMT gene sense, 5'-CTCTGGGT GAA GCT AAG ATTT-3' and anti-sense, 5'-CAT TTT CAAA CACCT GCTCG-3', which amplifies 116-bp PCR product. To verify the integrity of cDNA, β2-microglobulin expression was also analysed using the primer sequences sense, 5'-CATCAGGGT A CCCC AAGA-3' and anti-sense, 5'-GACA AATGCA TGTCGC CAC-3', which amplifies 165-bp PCR product. These two sets of primers span junctions between two exons, so amplification of the contaminating genomic DNA can be excluded. PCR amplification was performed for 94°C for 10 min, then 35 cycles of 94°C for 45 s, 57°C for 60 s and 72°C for 30 s, followed by a final elongation step at 72°C for 10 min.

SYK gene

The following primer sequences were used for SYK gene sense, 5'-TGCTCAAGGA AAAAC AGC TAC TAG-3' and anti-sense, 5'-CAC CAGC TCT CAG CTG TAT-3', which amplifies 507-bp PCR product. In this PCR, β2-microglobulin was also used as an internal control. The optimised PCR program was 94°C for 5 min, then 35 cycles of 94°C for 40 s, 62°C for 40 s and 72°C for 45 s, followed by a final elongation step at 72°C for 7 min. The PCR products were subjected to electrophoresis in 2.0% agarose gel and visualised by ethidium bromide staining.

RESULTS

Cytogenetic analysis performed on these tumours exhibited a 46,XX normal karyotype in 33 tumours. However, trisomy 14 and monosomy 22 were found in only 10 tumours (Table 2).

MGMT, CDH1, RAR-β and SYK promoter hypermethylation

The methylation status of ovarian tumours (GCTs) was determined at the respective loci using MSP. DNA from 43 ovarian tumours was modified using sodium bisulphite, which converts all unmethylated cytosine residues to uracil, but leaves methylated cytosines unchanged. To confirm that the modification was successful, all samples were first amplified with primers specific for unmethylated DNA at the p16 or MLH1 loci (even tumours methylated at these loci would be expected to be positive due to contamination with normal tissue, which is known to be unmethylated). The samples were next subjected to MSP using primers specific for methylated DNA at the respective loci being studied.

At least one of these four genes showed aberrant methylation in 17 tumour samples (17 out of 43). Methylation of only one gene was found in 13.95% (6 of 43) of tumours. The percentage of the tumours with methylation in genes 2, 3 and 4 is 9.3% (4 of 43), 9.3% (4 of 43) and 6.97% (3 of 43), respectively (Figure 1). Three tumours were found to be hypermethylated for four tumour suppressor genes (Table 2) investigated in the present study. The representative examples of MSP are shown in Figure 2.

We compared hypermethylation of MGMT, CDH1, RAR-β and SYK promoters in ovarian carcinoma (OC). As shown in Table 3, we found concordant hypermethylation of MGMT and CDH1 promoters (0.001), and MGMT and RAR-β promoters (0.0421). Similarly, hypermethylation of CDH1 and RAR-β promoters (0.0005) was also found. However, we found no correlation between the methylation status of MGMT and SYK promoters, CDH1 and SYK promoters or RAR-β and SYK promoters. There was no significant correlation between the DNA methylation status and clinical/pathological parameters with respect to age, tumour size and pathological grading.

RT-PCR for RAR-β, RAR-β2, MGMT and SYK gene expression study

To examine the biological role of promoter hypermethylation in GCTs of ovaries, we assessed the levels of gene expression by semiquantitative RT–PCR in tumours with known methylation status in RAR-β gene. Both RAR-β and RAR-β2 were found to have negative expression of mRNA in seven patients with OC showing promoter hypermethylation in RAR-β gene. Another patient was essentially negative for RAR-β2 mRNA, although it shows aberrant methylation in the RAR-β promoter region. All other patients of OC patients showed normal expression of both RAR-β and RAR-β2 and were not methylated (Table 2). The data suggest that promoter hypermethylation leading to gene silencing may affect a variety of key pathways in germ cell tumorigenesis. Aberrant promoter methylation changes that occur in cancer are associated with
transcriptional repression and loss of function of the gene by interrupting the binding of proteins involved in the transcription activator complex. Our gene expression analysis by RT–PCR demonstrated that all tumours that showed methylation of RAR-\(b\) genes also showed downregulation of mRNA levels in the methylated tumours. Thus in these cases, promoter hypermethylation is one mechanism whereby gene expression can be deregulated in GCTs. We also assessed the levels of gene expression in MGMT and SYK genes by semiquantitative RT–PCRs in these patients. Only three patients showed negative expression of mRNA in these genes. Two patients showed promoter hypermethylation in all four tumour suppressor genes and negative expression of mRNA in RAR-\(b\), MGMT and SYK.

**DISCUSSION**

Monosomy 22 and trisomy 14 appear to be emerging as nonrandom chromosome abnormalities in this type of tumours,
although it is also associated with other complex chromosome abnormalities. In light of the data, the present finding of monosomy 22 as the sole chromosome change in these tumours suggests that this karyotypic change, possibly followed by the acquisition of an extra chromosome 14 (trisomy), may be an early, nonobligatory event of the tumorigenesis of GCTs of ovarian origin. We found hypermethylation of MGMT in 32.5% cases of OC (14 out of 43). There are reports in the literature regarding the promoter hypermethylation of CDH1 gene in 20.9% OCs (9 out of 43). Hypermethylation of CDH1 gene and its reduced expression in gut, liver, prostate and breast cancers, which could be due to the disruption of intercellular adhesion and impairment of β-catenin mediated transactivation of cadherin–catenin complex (Griff et al, 1995; Sadot et al, 1998; Tamura et al, 2000; Corn et al, 2001; Oue et al, 2002), has been reported. DNA methylation in the RAR-β promoter region has been found in 18.6% ovarian tumours (8 out of 43). Methylation in the promoter region of RAR-β gene is frequently found to be associated with downregulation/loss of its expression in tissues affected with gastric carcinoma and in gastric cell lines.

Table 3 Correlation of promoter methylation of MGMT, CDH1, RARb and SYK in ovarian carcinomas

|                  | Methylated | Unmethylated | P-value |
|------------------|------------|--------------|---------|
| MGMT methylation status |            |              |         |
| CDH1 methylation status |          |              |         |
| Methylated       | 9          | 0            | 0.001*  |
| Unmethylated     | 5          | 11           |         |
| RAR-β methylation status |         |              |         |
| Methylated       | 7          | 1            | 0.0421* |
| Unmethylated     | 7          | 10           |         |
| SYK methylation status |         |              |         |
| Methylated       | 4          | 2            | 0.6609  |
| Unmethylated     | 10         | 9            |         |
| CDH1 methylation status |         |              |         |
| RAR-β methylation status |         |              |         |
| Methylated       | 7          | 1            | 0.0005* |
| Unmethylated     | 2          | 15           |         |
| SYK methylation status |         |              |         |
| Methylated       | 3          | 4            | 1       |
| Unmethylated     | 6          | 11           |         |
| RAR-β methylation status |         |              |         |
| SYK methylation status |         |              |         |
| Methylated       | 2          | 5            | 1       |
| Unmethylated     | 6          | 12           |         |

*Significant at P < 0.05.
head and neck cancers, breast cancer and non-small-cell lung
carcinoma (Xu et al., 1994, 1997; Hayashi et al., 2001; Oue et al.,
2002). DNA hypermethylation in SYK gene has been found in
16.3% ovarian tumours (7 out of 43). DNA methylation and loss of
expression has been reported in breast cancer (Coopman et al.,
2000; Yuan et al., 2001). It could be associated with DNA
hypermethylation mediated by methyl-CpG binding proteins that
bind to methylated cytosines and forms a repressive and inactive
complex of proteins that helps in repressing the transcription
including that of HDACs and are gene silencing (Wade et al., 1999).
Similar type of results have been reported for hMLH1 gene in
primary gastric cancers and endometrial cancers (Fleisher et al.,
1999; Leung et al., 1999). Several genes including tumour suppressor
and DNA repair genes such as hMLH1, RB1, VHL, p15, p16, RASSF1A, MGMT and
BRCA1 in human cancers (Herman et al., 1994, 1996, 1998; Merlo et al., 1995; Esteller et al.,
1999a; Baldwin et al., 2000; Gras et al., 2001; Nakayama et al., 2001;
Yoon et al., 2001) were shown to be epigenetically inactivated by
DNA methylation in tumours. Hypermethylation of CpG islands of
another tumour suppressor gene RASSF1A has been reported in
ovarian and renal cell carcinoma (Yoon et al., 2001). One of the
major mechanisms of tumour progression is thought to be the
inactivation of tumour suppressor genes. This inactivation can be
induced by mechanisms such as chromosomal deletion and loss of
function mutation in the coding region of genes or by epigenetic
alteration in the form of methylation of promoter regions. We
found that 15 of the MSI-positive tumours had hypermethylation
of either p16, BRCA1 or RASSF1A gene, whereas none of the MSI-
negative tumours (MSS) demonstrated the promoter hypermethy-
lation and this could be due to the epigenetic inactivation of either
p16, BRCA1 or RASSF1A genes (unpublished data). While it is
possible that most of the methylation events we observed
contribute in some way to the carcinogenic process, it is unlikely
that all the methylated loci play a direct role in ovarian
malignancies. Rather, methylation of only certain classes of
genes, such as tumour suppressor genes may be crucial to cancer
progression.

We found concordant hypermethylation in promoters of MGMT
and CDH1, MGMT and RAR-β, CDH1 and RAR-β. Retinoic acid
(RA) has profound regulatory effects in the control of many
biological processes such as development, immunomodulation,
differentiation, proliferation, reproduction and apoptosis (Lotan,
1980). The regulation of cell growth and differentiation of normal,
premalignant and malignant cells by retinoids is thought to result
from the direct and indirect effects of retinoids on gene expression.
These effects are mediated by the nuclear receptors, including
RAR-β2 located at 3p24. Retinoic acid receptor beta has four
alternative splicing forms and the β-2 form appears to possess
tumour-antagonizing activity at least in some cancers. There are
no reports of the mutations in the RAR-β gene, but it undergoes
epigenetic inactivation by promoter methylation in tumours of
different origin. Retinoic acid receptor beta underwent DNA
promoter region methylation in significant percent in several other
tumours such as lung (Virmani et al., 2000), cervical (Yang et al.,
2001) bladder (Maruyama et al., 2001), and prostate (Maruyama
et al., 2002). Squamous cell carcinomas and premalignant
dysplasias demonstrate a specific downmodulation of RAR-β
that can be restored by systemic retinoid therapy (Lotan, 1995). It has
also been reported that RA induces upregulation of CDH1
expression and the morphological change of fibroblastoid to
epithelioid growth of human pancreatic cancer cell line SUIT-2
(Jimmie et al., 1998). Promoter hypermethylation in three genes
MGMT, CDH1 and RAR-β together in seven cases of OC, which
also show loss of expression of both RAR-β and RAR-β2, may be
due to the involvement of the signalling pathway mediated by β-
catenin and other factors in the tumorigenic process in these OCs.
It has already been reported that the reduced expression of E-
 cadherin gene and its hypermethylation (Tamura et al., 2000),
and the reduced expression of RAR-β due to the promoter hyper-
methylation may be associated with the reduced expression of
CDH1. E-cadherin is involved in a signalling pathway mediated by
β-catenin and lymphocyte enhancer factor and T cell transcription
factors and can, therefore, explain the concordant hypermethyla-
tion in the promoter region of these three genes. Promoter
hypermethylation of RAR-β2 may block or interfere with the
retinoid signalling pathways in OC. Deregression of this pathway
results in constant activation of β-catenin, lymphocyte enhancer
factor and T cell factor target genes, including c-myc and cyclin D1.
For the cyclin-dependent kinase inhibitor p16, the silencing of the
gene mediated by promotor hypermethylation seems to be an early
event in the development and progression of the tumorigenic
process in ovarian cancers. CpG methylation patterns are
replicated together with DNA replication during the S phase
(Stein et al., 1982), and the altered transcriptional regulation via
aberrant promoter methylation could play an important and
significant role in the tumorigenic process of these ovarian
cancers. Our results suggest promotor methylation of tumour
suppressor genes such as MGMT, CDH1 and SYK. We also
analysed the relationship between promotor methylation status
and basal expression levels of RAR-β, RAR-β2, MGMT and SYK in
GCTs of ovaries. Our data clearly show that methylation in the
promoter region of RAR-β gene is correlated with downregulation/
loss of RAR-β2 gene expression of GCTs of ovaries. Therefore,
it might be the only mechanism responsible for loss of or
downregulation of RAR-β gene expression in this type of cancer
and plays a critical role in the tumour growth and development,
which can cause disruption in the retinoid signalling pathway,
the metastasis-related process and DNA repair processes in OC.
Similarly, out of 14 patients who show promotor hypermethylation
in MGMT, three show loss of expression while out of 7 patients
who show promotor hypermethylation in SYK gene, only three
were found to have loss of expression. It is clear from these results
that it is not always true for all the tumour suppressor genes to
show a positive correlation between promotor hypermethylation
and loss of expression in cancers.

There exist two different pathways that can contribute to the
development of cancers; genome-wide hypomethylation may lead
to the loss of chromosomes thus leading to chromosomal
instability, whereas promotor methylation in certain tumour
suppressor genes, which is responsible for gene silencing, can
lead to the development of cancers in somatic cells. Therefore,
the balance in DNA methylation is very important, and alteration
in these may be protective in one pathway but deleterious in the
other. It can therefore be concluded that promotor hypermethyla-
tion of MGMT, CDH1, RAR-β and SYK genes, and loss of
expression of RAR-β and RAR-β2 in ovarian cancers (GCTs) is
relatively common and this may also be useful as a tumour
marker for early diagnosis and subsequent disease monitoring. Hence,
these epigenetic signatures could play a decisive role in designing
treatment options for this category of ovarian cancer.

REFERENCES

Bae SI, Lee HS, Kim SH, Kim WH (2002) Inactivation of O6-methylguanine-
DNA methyltransferase by promoter CpG island hypermethylation in
gastric cancers. Br J Cancer Res 86: 1888 – 1892

Baldwin RL, Nemeth E, Tran H, Shvartsman H, Cass I, Narod S, Karlan BY
(2000) BRCA1 promoter region hypermethylation in ovarian carcinoma:
a population-based study. Cancer Res 60: 3329 – 3333.

© 2004 Cancer Research UK

British Journal of Cancer (2004) 90(4), 874 – 881
Chambon P (1996) A decade of molecular biology of retinoic acid receptors. FASEB J 10: 940 – 954

Chu DH, Morita CT, Weiss A (1998) The Syk family of protein tyrosine kinases in T-cell activation and development. Immunol Rev 165: 167 – 180

Coopman PJ, Do MT, Barth M, Bowden ET, Hayes AJ, Basyuk E, Blancato JK, Vezza FR, McLeskey SW, Mangeat PH, Mueller SC (2000) The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature (London) 406: 742 – 747

Corn PG, Heath EL, Heitmiller R, Fogt F, Forastiere AA, Herman JG, Wu TT (2001) Frequency of methylation of the 5` CpG island of E-Cadherin in esophageal adenocarcinoma. Clin Cancer Res 7: 2765 – 2769

Dal Cin P, Qi H, Pauwels P, Backa C, Van den Berge H (1997) Monosomy 22 in a fibrothecoma. Cancer Genet Cytogenet 99: 129 – 131

de The H, Vivanco-Ruiz MM, Tiollais P, Stunnenberg H, Dejean A (1990) Indentification of a retinoic acid response element in the retinoic acid receptor b gene. Nature (London) 343: 177 – 180

Duesberg P, Li R, Rasmick D, Rausch C, Willer A, Kraemer A, Yerganian G, Hehman R (2000) Aneuploidy precedes and segregates with chemical carcinogenesis. Cancer Genet Cytogenet 119: 83 – 93

Eden A, Gandet F, Waghmare A, Jaensch R (2003) Chromosomal instability and tumour promoted by DNA hypomethylation. Science 300: 455

Esteller M, Catasus L, Matias-Guiu X, Peinado MA, Watkins DN, Issa JP, Sidransky D, Baylin SB, Herman JG (1999a) hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. Am J Pathol 155: 1767 – 1772

Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999b) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 59: 793 – 797

Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Hamilton SR, Burger PC, Baylin SB, Herman JG (2000) Inactivation of the DNA repair gene O6-Methylguanine-DNA Methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res 60: 2368 – 2371

Evan MF, McDicken IW, Herrington CS (1999) Numerical abnormalities of chromosomes 1, 11, 17, and X are associated with stromal invasion in serous and mucinous epithelial ovarian tumours. J Pathol 189: 53 – 59

Fleisher AS, Esteller M, Wang S, Tamura G, Suzuki M, Ohwada M, Saga Y, Ochiai K, Sato I (2000) DNA replication error by promoter hypermethylation is associated with G to A mutations in hMLH1 gene promoter in human gastric cancers with microsatellite instability. Cancer Res 60: 1850 – 1855

Gandet F, Hodgson JG, Eden A, Jackson-Grusby L, Daussman J, Gray JW, Fleisher AS, Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999a) hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. Am J Pathol 155: 1767 – 1772

Gerber JT, Mohgal N, Frangioni JV, Sugerbaker DJ, Neel BG (1991) High frequency of retinoic acid receptor b2 abnormalities in human lung cancer. Oncogene 6: 1859 – 1868

Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE, Baylin SB (1995) E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 55: 5195 – 5199

Gras E, Catasus L, Arguelles R, Gamallo C, Garcia del Muro X, Torregrosa A, Munoz J, Castellsague X, Condom E, Gandet F, Hodgson JG, Eden A, Jackson-Grusby L, Daussman J, Gray JW, Fleisher AS, Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (2000) Aneuploidy precedes and segregates with chemical carcinogenesis. Cancer Genet Cytogenet 119: 83 – 93

Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Wilson JK, Hamilton SR, Kizlter KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci USA 95: 6870 – 6875

Jimi S, Shono T, Tanaka M, Kono A, Yamada Y, Sudo K, Kuwano M (1998) Effect of retinoic acid on morphological changes of human pancreatic cancer cells on collagen gels: a possible association with the metastatic potentials. Oncol Res 10: 7 – 14

Leung SY, Yuen ST, Chu KM, Chan AS, Ho JC (1999) hMLH1 promoter methylation and lack of hMLH1 expression in sporadic gastric carcinomas with high-frequency microsatellite instability. Cancer Res 59: 159 – 164

Lotan R (1999) Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochim Biophys Acta 1605: 33 – 91

Lotan R (1995) Retinoids and apoptosis: implications for cancer chemoprevention and therapy. J Natl Cancer Inst 87: 1655 – 1657

Lotan R, Xu C, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK (1995) Suppression of retinoic acid receptor beta in premalignant oral lesions and its up-regulation by isotretinoin. N Engl J Med 333: 1405 – 1410

Maruyama R, Toyooka S, Toyooka KO, Harada K, Virmani AK, Zochbauer-Muller S, Farinas AJ, Vakar-Lopez F, Minna JD, Sakagowsky A, Czerniak B, Gazdar AF (2001) Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. Cancer Res 61: 8659 – 8663

Matias-Guiu X, Toyooka S, Toyooka KO, Virmani AK, Zochbauer-Muller S, Farinas AJ, Minna JD, McConnell J, Frenkel EP, Gazdar AF (2002) Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 8: 514 – 519

Merlo A, Herman JG, Mao L, Lee DJ, Gabreilson E, Burger PC, Baylin SB, Sidransky D (1995) 5-Cpg island methylation is associated with transcriptional silencing of the tumour suppressor p16CDKN2/MTS1 in human cancers [see comments]. Nat Med 1: 686 – 692

Nakayama K, Takebayashi Y, Namiki T, Tahamashi N, Nakayama S, Uchida T, Miyazaki F, Fukumoto M (2001) Comprehensive allelotype study of ovarian tumours of low malignant potential: potential differences in pathways between tumours with and without genetic predisposition to invasive carcinoma. Int J Cancer 94: 605 – 609

Oue N, Motoshita J, Yokozaki H, Hayashi A, Tahara E, Taniyama K, Matususaki K, Yasui W (2002) Distinct promoter hypermethylation of p16INK4A, CDH1 and RAR-beta in intestinal, diffuse-adherent, and diffuse-scattered type gastric carcinomas. J Pathol 198: 55 – 59

Rosas SL, Koch W, da Costa Carvalho MG, Wu L, Califano J, Westra W, Jen J, Sidransky G (2001) Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res 61: 939 – 942

Russel P, Bannatyne P (1989) Surgical Pathology of Ovaries. London: Churchill Livingstone.

Sados E, Simcha I, Shuttman M, Ben-Zee’ev A, Geiger B (1998) Inhibition of beta-catenin-mediated transactivation by cadherin derivatives. Proc Natl Acad Sci USA 95: 15339 – 15344

Sandberg AA (1991) Chromosome abnormalities in human cancer and leukaemia. Mutat Res 247: 231 – 240

Seewaldt VL, Johnson BS, Parker MB, Collins SJ, Swisshelm K (1995) Expression of retinoic acid receptor-beta mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. Cell Growth Differ 6: 1077 – 1088

Speelman F, Dermaut B, De Potter CR, Van Gele M, Van Roy N, De Paepe A, Laureys G (1997) Monosomy 22 in a mixed germ cell-sex cord-stromal tumor of the ovary. Genes Chromosomes Cancer 19: 192 – 194

Stein R, Gruenbaum Y, Pollack Y, Rajin A, Cedar H (2001) Clonal inheritance of the pattern of DNA methylation in mouse cells. Proc Natl Acad Sci USA 79: 61 – 65

Suzuki M, Ohwada M, Saga Y, Ochiai K, Sato I (2000) DNA replication error is frequent in ovarian granulosa cell tumours. Cancer Genet Cytogenet 122: 55 – 62

Takeichi M (1995) Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 7: 619 – 627

Tamura G, Yin J, Wang S, Fleisher AS, Zou T, Abraham JM, Dong R, Smolinski KN, Wilson KT, James SP, Silverberg SG, Nishizuka S, Terashima M, Toyama Y, Meltzer SJ (2000) E-cadherin gene promoter hypermethylation in primary human gastric carcinomas. J Natl Cancer Inst (Bethesda) 92: 569 – 573
Van den Berghe I, Dal Cin P, De Groef K, Michielsen P, Van den Berghe H (1999) Monosomy 22 and trisomy 14 may be early events in the tumorigenesis of adult granulosa cell tumor. Cancer Genet Cytogenet 112: 46–48

Virmani AK, Rathi A, Zochbauer-Muller S, Sacchi N, Fukuyama Y, Bryant D, Maitra A, Heda S, Fong KM, Thunnissen F, Minna JD, Gazdar AF (2000) Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst 92: 1303–1307

Wade PA, Gegonne A, Jones PL, Ballester E, Aubry F, Wolfe AP (1999) Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet 23: 62–66

Xu X-C, Ro JY, Lee JS, Shin DM, Hong YK, Lotan R (1994) Differential expression of nuclear retinoid receptors receptors in normal, premalignant and malignant head and neck tissues. Cancer Res 54: 3580–3587

Xu X-C, Sneige N, Liu X, Nandagiri R, Lee JI, Lukmanji F, Hortobagyi G, Lippman SM, Dhingra K, Lotan R (1997) Progressive decrease in nuclear retinoic acid receptor b messenger RNA levels during breast carcinogenesis. Cancer Res 57: 4992–4996

Yang Q, Mori I, Shan L, Nakamura M, Nakamura Y, Utsunomiya H, Yoshimura G, Suzuma T, Tamaki T, Umemura T, Sakurai T, Kakeji K (2001) Biallelic inactivation of retinoic acid receptor beta2 gene by epigenetic change in breast cancer. Am J Pathol 158: 299–303

Yoon JH, Dammann R, Pfeifer GP (2001) Hypermethylation of the CpG islands of the RASSF1A gene in ovarian and renal cell carcinomas. Int J Cancer 94: 212–217

Yoshiura K, Kanai Y, Ochiai A, Shimoyama Y, Sugimura T, Hirohashi S (1995) Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci USA 92: 7416–7419

Yuan Y, Mendez R, Sahin A, Dai JL (2001) Hypermethylation leads to silencing of the SYK gene in human breast cancer. Cancer Res 61: 5558–5561

Zheng Z, Pan J, Chu B, wong YC, Cheung AL, Tsao SW (1999) Down-regulation and abnormal expression of E-cadherin and β-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. Hum Pathol 30: 458–466