The Contribution of Genetic and Epigenetic Changes in Granulosa Cell Tumors of Ovarian Origin

Varinderpal S. Dhillon, Mohammad Aslam, and Syed Akhtar Husain
Cytogenetics Laboratory, Department of Biosciences, Jamia Milia Islamia, New Delhi, India

ABSTRACT

Purpose: Granulosa cell tumors (GCTs) are relatively rare and are subtypes of the sex-cord stromal neoplasms. A better understanding of the molecular genetics underlying various steps in malignant transformation is critical to success in the battle against this disease. Changes in the status of methylation, known as epigenetic alterations, are one of the most common molecular alterations in human cancers, including GCTs. Chromosomal instability and microsatellite instability (MSI) are common in these GCTs. We tested the hypothesis that C→T transition polymorphism in the promoter region of cytosine DNA-methyltransferase-3B (DNMT3B) and its altered expression are also associated with hypermethylation of the genes. We also attempted to determine the relationship between MSI of ovarian carcinoma and hMLH1 hypermethylation in these tumors.

Experimental Design: We studied chromosome instability in 25 GCTs by detecting gross chromosome rearrangements in cultured peripheral blood lymphocytes. MSI was assessed using six microsatellite markers (BAT25, BAT26, D25S123, DSS346, D11S1318, and D17S250). Using sensitive methylation-specific PCR, we searched for aberrant promoter hypermethylation in a panel of genes including p16, BRCA1, RASSF1A, ER-α, TMS1, TIMP3, Twist, GSTP1, AR, and hMLH1. Polymorphism in the DNMT3B gene was assessed by the PCR-RFLP method, and DNMT3B expression was studied by reverse transcription-PCR assay.

Results: Chromosome instability was indicated by significantly higher frequencies of chromosome aberrations (6.24%; P < 0.001) compared with controls (2.12%). The most frequently observed changes include trisomy 14 and monosomy 22. MSI has been found in 19 of 25 tumors, and loss of heterozygosity has been found in 9 of 25 tumors. Frequencies of methylation in GCTs were 40% for p16 and ER-α; 36% for BRCA1 and RASSF1A; 28% for hMLH1; 24% for TIMP3, Twist, and GSTP1; and 20% in TMS1 and AR. TT genotype was found only in two cases; the remainder were either CC or CT type. There was no significant alteration in the expression of DNMT3B in these patients.

Conclusions: Coexistence of chromosome instability, MSI, and hypermethylation suggests that both genetic and epigenetic mechanisms may act in concert to inactivate the above-mentioned genes in these GCTs. These mechanisms can be an early event in the pathogenesis of these tumors, and it can be a critical step in the tumorigenic process. All these events might play an important role in early clinical diagnosis and in chemotherapeutic management and treatment of the disease. Larger studies may lend further understanding to the etiology and clinical behavior of these tumors.

INTRODUCTION

Ovarian cancer is the one of the most common cancer in women and causes more deaths in the United States and Europe than any other female reproductive organ cancer. Granulosa cell tumors (GCTs) are relatively rare, accounting for approximately 5% of all ovarian cancers. GCTs are classified as a subgroup of sex-cord stromal neoplasms, together with Sertoli cell tumors and Leydig cell tumors. The etiology of GCTs remains unclear. Trisomy 12 occurs in the juvenile variant of GCT, whereas monosomy 22 frequently associated with trisomy 14 appears to be the most common karyotypic abnormality found in the adult-type variant (1). It is now accepted that accumulation of multiple genetic aberrations can lead to the development of most carcinomas. Abnormalities of chromosomes 1, 3, 6, 11, 12, 15, 17, and X are found most frequently in cancer patients (2). 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 (3). Each alteration may be mediated through gross chromosomal changes and hence has the potential to be detected cytogenetically (4). Chromosomal instability, characterized by an abnormal number or structure of chromosome(s), is a common feature of many human cancers such as skin cancers, primary neoplasias, Hodgkin’s lymphoma, bladder cancer, breast cancer, carcinoma of the cervix uteri, and prostate gland cancer (4, 5). However, the mechanisms behind these changes are still unclear.

Alterations in DNA methylation appear to play a key role in a number of diseases, particularly in cancer, in which inactivation of many genes known to be important in tumor development has been found to be associated with hypermethylation in the promoter-associated CpG islands. There are many reports in the literature regarding the hypermethylation of normally unmethylated, promoter region CpG islands and their association with transcriptional inactivation of several tumor suppressor genes [hMLH1, VHL, p15, p16, BRCA1, and RASSF1A in human cancers (6–14)]. Recent studies have linked this aberrant de novo methylation of CpG islands to overexpression of...
DNMT3A and DNMT3B. Both DNMT3A and DNMT3B are required for the establishment and maintenance of genomic methylation patterns and proper murine development. Both genes are up-regulated in cancers, including cancers of the bladder, colon, kidney, and pancreas, to different degrees (15–17). A recently found C→T single-base transition polymorphism in a novel promoter of DNMT3B, 149 bp down the transcription start site, can significantly increase the promoter activity of the DNMT3B gene (18).

Therefore, all of the above-mentioned findings led us to evaluate the frequency of chromosome instability, microsatellite instability (MSI), and loss of heterozygosity (LOH) in GCTs of ovarian origin. To determine the role of aberrant methylation in this type of cancer, we have studied these tumors at multiple loci using methylation-specific PCR (MSP). We also hypothesized that the polymorphism in DNMT3B and its altered expression may be associated with regulation of the methylation status of multiple genes in this cancer.

MATERIALS AND METHODS

Cytogenetic Study. The subjects were 25 patients affected with GCTs of ovarian origin without a positive family history. All of them were untreated at the time of study. For comparison, 50 normal individuals ranging in age from 20 to 60 years were studied simultaneously under similar experimental conditions. The tumor grading was as follows: FIGO (International Federation of Gynecologists and Obstetricians) stage IA, 11 patients; FIGO stage IB, 10 patients; and FIGO stage IC, 4 patients. All these diagnoses were reviewed by a gynecologic pathologist, and the tumors were assessed using standard criteria (19). This study was approved by the Health Research Ethics Board of the Faculty of Natural Sciences, Jamia Millia Islamia. Informed consent was provided by all of the patients before the study.

Peripheral blood samples were cultured for 72 h in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (heat inactivated; GIBCO) and 0.06 μg/ml phytohemagglutinin (GIBCO). Colcemid (0.06 μg/ml) was added 30 min before harvesting the cultures. G-banding technique was applied to identify chromosome aberrations. One hundred metaphases were karyotyped (except six patients) to identify the types of various chromosome aberrations. The common chromosome aberrations were generally classified as structural or numerical. Structural aberrations included translocations, inversions, deletions, insertions, and amplification, whereas numerical aberrations included loss or duplication of the whole chromosome.

Molecular Genetic Study. Twenty-five paraffin-embedded ovarian tumors were selected for the present study. For MSI analysis, tumor and normal DNA were extracted using the Puragene cell and tissue kit with few modifications. Matched normal and tumor DNA were analyzed for MSI using a panel of different markers (BAT25, BAT26, D2S123, D5S346, D11S1318, and D17S250) as recommended by the National Cancer Institute (NCI). DNA derived from normal or tumor tissues was amplified by PCR in 10-μl reaction mixtures containing 2 μmol deoxynucleotide triphosphates, 1 unit of AmpliTaq polymerase, 1 ml of 10× PCR buffer (Roche), and 1–1.5 μl of 1.25 mm primer pairs. Reactions were carried out beginning with a 95°C denaturing step for 5 min. Subsequently, each cycle began at 95°C for 45 s, with each cycle having a 72°C extension of 1 min. Three different annealing temperatures were used for the reaction [68°C × 5 cycles (5 min each), 58°C × 5 cycles (2 min each), and 56°C × 25 cycles (2 min each)] followed by a final extension of 72°C for 10 min. One of each of the paired primers was labeled with fluorescent dye. Fluorescence-labeled PCR products were detected using ABI 310 Genetic Analyzer. The following capillary electrophoresis run parameters were used: 5-s injection time; 15.0 kV; 60°C; and 24 min electrophoresis time. MSI was identified when shifts were noted in the allelic band(s) in DNA compared with normal DNA consistently in repeated experiments. If one of the alleles is missing, it was considered LOH positive. χ² and Fisher’s exact test were used to determine differences between variables using SPSS (SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant.

Methylation-Specific PCR. DNA methylation patterns in CpG islands of tumor suppressor genes p16 (20), BRCA1 (21), RASSF1A (22), ER-α (23), TMS1 (24), TIMP and GSTP1 (25), Twist (26), AR (27), and hMLH1 (28) were determined by chemical modification with sodium bisulfite. The primer sequences used have all been reported previously and can be found in the report referenced after each gene. Briefly, 1 μg of DNA 1 was denatured by NaOH (50 μl; final concentration, 0.2 M) for 10 min at 37°C. One μg of salmon sperm DNA (Sigma, St. Louis, MO) was added as carrier before modification. Freshly prepared hydroquinone (30 μl; 10 mM; Sigma) and 520 μl of sodium bisulfite [3 M (pH 5.0); Sigma] were mixed, and samples were incubated under mineral oil at 55°C for 16 h. The DNA samples were desalted through Wizard columns (Promega, Madison, WI) and then desulfonated by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by EtOH precipitation. DNA was resuspended in water and used immediately or stored at −20°C. Fifty to 100 μl of bisulfite-modified DNA were used for each MSP.

CpG islands in the above-mentioned genes were examined by MSP. Forward and reverse primers (primer sequences and PCR conditions are available on request) that corresponded to the predicted sequence of methylated or unmethylated genomic DNA after sodium bisulfite treatment were synthesized. For the reaction, 50 ng of sodium bisulfite-treated DNA were added to reaction buffer containing 1.25 mm deoxynucleotide triphosphate, 16.6 μm (NH₄)₂SO₄, 67 mM Tris (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, 0.1% DMSO, 10 pmol of forward and reverse primers specific to the methylated and methylated DNA sequences, and 2.5 units of AmpliTaq Gold (PE Biosystems, Foster City, CA). Conditions were 95°C for 5 min, followed by 35 or 40 cycles of 95°C for 30 s, 45–65°C for 40 s, and 72°C for 45 s, with a final extension cycle of 72°C for 5 min. DNA isolated from normal peripheral lymphocytes from healthy individuals served as a negative methylation control. Human placental DNA was treated in vitro with SssI methyltransferase (New England BioLabs, Inc., Beverly, MA) to create completely methylated DNA at all CpG-rich regions and served as a positive methylation control. MSP products were analyzed by 3% agarose gel electrophoresis with ethidium bromide staining. A positive control and a negative control were included in each amplification reaction.
**DNMT3B Gene Polymorphism.** PCR-RFLP assay (29) was used to identify C→T transition at nucleotide –149 (C463597; Genbank accession no. AL035071) in the promotor region of DNMT3B using primers 5′-gcttgacagccgacagc-3′ (nucleotides 46151–46170) and 5′-ggtgcgegcaactccaggc-3′ (nucleotides 46530–46511) that amplify a 380-bp target DNA fragment. This fragment spans the upstream region and the first exon of the DNMT3B gene. PCR mixture (20 μl) contained 50–100 ng of genomic DNA, 1 X PCR buffer, 12.5 pmol of each primer, 0.1 mM each deoxynucleotide triphosphate, 1.5 mM MgCl₂, and 1.25 units of Taq polymerase (Sigma). The PCR profile consisted of an initial denaturation step of 95°C for 5 min and 35 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s, followed by a final extension step of 72°C for 10 min. The 380-bp fragment was then digested with AvrII (New England BioLabs, Inc.) for 16 h at 37°C. The digested products were separated on a 2.5% agarose (Sigma) gel containing ethidium bromide. The wild-type C allele lacked the AvrII restriction site and therefore produced a single 380-bp band, whereas variant T allele that had an AvrII restriction site resulted in two bands of 207 and 173 bp.

**Reverse Transcription-PCR for DNMT3B.** DNMT3B mRNA levels in cancer patients were compared with DNMT expression in normal ovarian cells using semiquantitative reverse transcription-PCR. In brief, cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Promega, Mannheim, Germany) and amplified in duplex reactions in a total volume of 25 μl containing 150 μM each deoxynucleotide triphosphate, 1.5 mM MgCl₂, 10 pmol of each primer pair, and 1.0 unit of Taq polymerase. After initial denaturation at 95°C for 5 min, 35 cycles of 30 s at 94°C, 30 s at 61°C, and 1 min at 72°C were performed, followed by a final 10-min elongation step at 72°C. The following primers were used to amplify DNMT3B: forward, 5′-gaacgggtgaggagcggaa-3’; and reverse, 5′-ggccctgtggaggacgaga-3’, which yields a 270-bp product. GAPDH1 was used as internal control. Each reaction was performed in triplicate. PCR products were electrophoresed on 2% agarose gels and quantified using a densitometer (Molecular Dynamics).

**Statistical Analysis.** Statistical differences were assessed with Fisher’s exact test by InStat software. Two-sided tests were used to determine significance. P values < 0.05 were regarded as statistically significant.

**RESULTS**

**Cytogenetic Study.** We observed significant increase (6.24%; P < 0.001) in aberrant metaphases in GCT patients as compared with age-matched control subjects (2.12%). Most of these aberrations were found to be constitutional in nature. No correlation of chromosome aberration with stage of cancer was found. However, in all cancer stages, the complex types of chromosomal aberrations were observed in patients rather than in controls. Most of the structural aberrations seen in controls were constitutional and were not due to any type of exposure to clastogenic agent/chemical. The most common structural abnormalities detected in these cancer patients were found in chromosomes 1, 3, 6, 7, 8, 11, 16, 17, and X. Chromosomes 1 and 3 were most frequently involved in translocations with other chromosomes, followed by chromosomes 11 and 17. Trisomy 14 and monosomy 22 were found quite frequently in these tumors. Other frequently observed structural changes included inversions of chromosomes 10 and 17. The most frequently observed deletion in these patients was deletion of X chromosome, followed by deletion of chromosome 17. The major structural and numerical aberrations/cell in cancer patients were found to be statistically significant (0.062; P < 0.001) as compared with controls (0.0018).

**Molecular Genetic Study.** DNA samples from 25 GCT of ovarian origin-matched blood samples were analyzed, using nine microsatellite markers that include five NCI designated markers. Tumors from patients OC001, OC003, OC004, OC005, OC006, OC008–OC0012, OC0015, OC0017, OC0018 and OC0020–OC0025 showed MSI at two or more of the NCI designated loci (Fig. 1A), whereas tumors from patients OC002, OC007, OC0013, OC0014, OC0016, and OC0019 were found to be microsatellite stable (MSS). The representative examples of MSI and LOH are shown in Fig. 1B. The frequency of instability at each of the microsatellite locus is shown in Fig. 2. Markers such as BAT25 and DSS346 were found to be most frequently altered in the present study, followed by BAT26, DS11S1318, D17S250, and D2S123. Eighteen (76%) of the tumors demonstrated microsatellite instability-high (MSI-H), whereas one (4%) showed MSI-low (MSI-L) in relation to five NCI designated markers. However when all of the markers were considered collectively, 19 tumors (76%) were found to be MSI-H. Markers BAT25 and BAT26 were also evaluated for polymorphic variation in the patient population. However, no polymorphic changes were detected at the BAT25 and BAT26 loci in our population. The pentanucleotide repeat motifs showed the lowest frequency as compared with mono- and dinucleotide repeats. LOH was detected in 11 patients. Eight tumors showed LOH with the D17S250 marker. LOH is generally defined as loss of preexisting alleles in the tumors as compared with normal alleles, and MSI is defined as a change of any length due to either insertion or deletion of repeating units in a microsatellite within a tumor as compared with normal tissue. No statistical significance was obtained when comparing MSI with age, histological type, and grade.

**Frequency of Hypermethylation in Different Genes.** Data for frequencies of methylation of the seven target genes are summarized in Fig. 2. In samples from GCTs, the methylation frequency of the target genes was 40% for p16 and ER-α; 36% for BRCA1 and RASSF1A; 28% for hMLH1; 24% for TIMP3, Twist, and GSTP1; and 20% in TMS1 and AR. Normal ovarian tissue could not be obtained to determine whether any of the loci exhibited methylation in nonneoplastic cells. However, in the 25 paired normal noncancerous samples adjacent to the tumor, values were 4% for p16, ER-α, and hMLH1; 2% for BRCA1 and RASSF1A; 3% for TIMP3, Twist, and GSTP1; and 5% in TMS1 and AR. DNA isolated from lymphocytes in these individuals also did not show any significant hypermethylation in any of these genes because it resulted only in faint bands (three samples), thus indicating a small fraction of infiltrating and circulating tumor cells or preneoplastic cells present in the normal sample. Alternatively, low levels of methylation cannot be ruled out in these patients. Fig. 3 illustrates representative examples of
methylation patterns in GCTs of ovarian origin. Promoter hypermethylation in the \textit{hMLH1} gene was noted in 7 of 19 tumors that showed MSI-H, whereas none of the MSS tumors showed any promoter hypermethylation in the \textit{hMLH1} gene, and there is no significant relationship between these two variables. No significant association of promoter hypermethylation was found in nine genes with different tumor grades, with the exception of AR (P = 0.0464; IA versus IB and IC; Table 1; Fig. 4). BRCA1 promoter methylation is found to be significantly associated (P = 0.0099) with LOH involving the D17S250 marker.

**Polymorphism in DNMT3B and Its Expression.** Nine individuals had the CC (wild-type) genotype, 14 had the CT (heterozygous) genotype, and 2 had the TT (mutant type) genotype. No clear-cut pattern of any specific association with tumor grades was found among these patients. Two tumors of the TT genotype also exhibited MSI-H (Figs. 1A and 5A); however, the sample size is too small to reach any conclusion, and it must be kept in mind that this type of cancer comprises only 5% of all ovarian cancers. Expression levels of the DNMT3B transcripts were determined by semiquantitative reverse transcription-PCR analysis. Expression of \textit{DNMT3B} mRNAs was readily detectable in all 25 tumors designated as MSI-H/L or MSS and in all of the genotypes (Fig. 5, B–D). DNMT3B mRNA expression levels in the cancerous cells relative to GADPH mRNA levels were determined. DNMT3B expression was observed in cancerous cells compared with normal (control) ovarian cells (mean expression level ± SE: normal ovarian cells, 1.46 ± 0.01; cancerous cells, 1.66 ± 0.02; stage IA, 1.62 ± 0.01; stage IB, 1.61 ± 0.02; stage IC, 1.92 ± 0.02). There is no significant increase in DNMT3B in the cancerous cells compared with normal ovarian cells. However, this increase is more prevalent (P < 0.05) in cancerous cells from stage IC.

**DISCUSSION**

The results indicated that the frequency of aberrant metaphases in patients with GCT is significantly higher than that in controls. If there is a continuous degree of chromosome instability in populations (30), it could be assumed that these patients...
show, to a certain extent, chromosome instability. The results indicate that ovarian cancer patients show chromosome instability that is lower than that found in Hodgkin’s lymphoma, bladder carcinoma, and prostate cancer but higher than that found in patients with carcinoma of cervix uteri and breast cancer (5, 31). The higher number of major structural and numerical aberrations in these patients further supports the above-mentioned hypothesis of chromosome instability. The structural and numerical changes found in these patients may be classified as constitutional aberrations. There are reports in the literature that suggest the presence of increased structural and numerical aberrations in families with higher incidences of neoplasia (30, 31). The patients with an unstable genome, as found in the present study, must be more prone to cancer or are also at high risk of developing certain other types of cancer. Cellular oncogenes such as int2 and cst1, which are localized on chromosome 11q13 and 11q23, respectively, are most commonly found to be involved in chromosome rearrangements (cancer specific) in the present study and are also found to be associated with hematological disorders (32). In the present study, the chromosome rearrangements, deletions, and gain or loss of chromosomes 1, 3, 7, 11, 13, 17, and X in some patients further support the hypothesis of Atkin and Baker (33). Tumor suppression in regions of these chromosomes must function properly to regulate growth in a number of tissues, and their inactivation must therefore be required in the development of multiple tumors. Although chromosomal instability is a common finding in malignant tumors, its precise pathogenetic role remains to be established. Rapid cellular proliferation would undoubtedly be of selective value in a developing tumor. It is likely that cells could increase their mitotic frequency by decreasing the cell cycle intervals spent on DNA repair. The continuous rearrangement of the chromosome complement may even impose a burden on the cell population, and efficient growth would be dependent on a delicate balance between proliferation rate and mutation rate. Functions that stabilize the chromosome complement while still allowing a permanent disruption of cellular checkpoints would then be highly favorable for efficient tumor development. Cancer cells must escape several levels of controls involving malignant transformation through multiple steps, each or most of which may be accompanied and/or mediated by specific chromosome changes. These patients might be at greater risk because of inherent chromosome instability, as evident in the present study, that could result in small populations of somatic cells with chromosome abnormalities. It is conceivable that some of these patients might have inherited a first hit as per multiple model of origin of cancers (34). These patients might therefore be at higher risk of developing secondary tumors because of acquired inherent instability. Chemotherapeutic agents and/or radiotherapy used for treatment and management of the cancer may also be responsible to

Fig. 2 Methylation profile of GCTs of ovarian origin in 25 patients for p16, BRCA1, RASSF1A, ER-α, TMS1, TIMP3, Twist, GSTP1, and AR genes. Results were scored as methylated (■) or unmethylated (□).

![Methylation profile of GCTs of ovarian origin in 25 patients for p16, BRCA1, RASSF1A, ER-α, TMS1, TIMP3, Twist, GSTP1, and AR genes. Results were scored as methylated (■) or unmethylated (□).](image-url)
increase chromosome (DNA) damage, which further supports the above-mentioned hypothesis regarding genomic instability.

These genotypic changes in cancer cells may be due to genomic instability, which appears to be a fundamental characteristic of cancer, acquired at the early steps of development. Multiple rearrangements of chromosomal structure are also a very common manifestation of instability in epithelial cancers. In addition to these, cytoskeletal defects and breakage-fusion-bridges cycles are also thought to be a possible source of chromosomal instability and karyotypic heterogeneity in cancer cells. Frequent generation of novel structural chromosomal rearrangements detected in subclones of human prostate cancer cell lines led to the suggestion that this chromosomal instability could be an intrinsic characteristic of cancer cells. Telomeric dysfunctions during growth crisis could also contribute to genomic instability during development. Restoration of telomere function stabilizes telomere ends and thus abrogates this cause of karyotypic lability, which may allow crisis resolution and continued growth. Mismatch repair-defective cells may be capable of bypassing this crisis by allowing telomeres to be replenished even in the absence of telomerase.

Table 1: Correlation of promoter methylation status of different genes in GCTs of ovarian origin in relation to hMLH methylation status and different histological grades

<table>
<thead>
<tr>
<th>Gene</th>
<th>methylation status</th>
<th>hMLH methylation status</th>
<th>Histological classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>M 2 8 0.6597</td>
<td>5 3 1 0.4341</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>BRCA1</td>
<td>M 5 10 0.0581</td>
<td>3 3 0.6766 0.1162</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>M 4 5 0.2049</td>
<td>3 4 0.6766 0.6016</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>ER-α</td>
<td>M 5 10 0.0752</td>
<td>3 4 0.4139 0.2668</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>TMS1</td>
<td>M 2 3 0.5968</td>
<td>3 1 1 0.6232</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>TIMP3</td>
<td>M 6 15 0.6372</td>
<td>2 3 0.6609 1</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>Twist</td>
<td>M 1 5 0.6372</td>
<td>2 3 0.5494</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>GSTP1</td>
<td>M 1 5 0.6372</td>
<td>3 1 1 1</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>AR</td>
<td>M 2 3 0.5968</td>
<td>0 3 2 0.464* 0.1664</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
<tr>
<td>hMLH1</td>
<td>M 3 3 1 1</td>
<td>1</td>
<td>IA 1 IB 1 IC 1</td>
</tr>
</tbody>
</table>

Abbreviation: M, methylated; U, unmethylated.

* Fisher’s exact test, versus methylation in hMLH.
† Fisher’s exact test, stage IA versus stage IB and IC.
‡ Fisher’s exact test, stage IA and IB versus stage IC.

Fig. 3 Representative examples of the MSP assay. Results of testing for the methylated forms of p16, BRCA1, ER-α, RASSF1A, TMS1, TIMP3, TWIST, GSTP1, AR, and hMLH1 are illustrated. U, unmethylated form; M, methylated form; Uc, control unmethylated DNA; Mc, control methylated DNA.
that DNA mismatch repair deficiency may contribute to the pathogenesis of ovarian cancer, and this deficiency may be an early event in the development and progression of the disease. 

MSI reflects alterations in the patterns of short tandem repeats that are highly polymorphic and distributed through the genome. MSI is implicated in the pathogenesis of colon, gastric, and endometrial carcinomas, which closely resembles the ovarian cancers (40). Quite a variable range of MSI is reported in the literature, and this could be explained by the various microsatellites analyzed and the various criteria used in those studies (41, 42).

One of the major mechanisms of progression is thought to be the inactivation of 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 19 MSI-positive tumors had hypermethylation of either of the $p16$, $BRCA1$, $RASSF1A$, $ER$-α, $TMS1$, $TIMP3$, $Twist$, $GSTP1$, $AR$, or $hMLH1$ gene, whereas none of the six MSI-negative tumors (MSS) demonstrated promoter hypermethylation. Our findings indicate that this could be due to the epigenetic inactivation of either of these genes. Similar results have been reported for the $hMLH1$ gene in primary gastric cancers and endometrial cancers (44). $TIMP-3$ is thought to suppress primary tumor growth, and its silencing through promoter hypermethylation may be responsible for tumor growth (45). Several genes, including suppressor genes and DNA repair genes ($p16$, $MGMT$, $VHL$, $MLH1$ and $BRCA1$), were shown to be epigenetically inactivated by DNA methylation in cancers. Hypermethylation of CpG islands of another tumor suppressor gene, $RASSF1A$, has been reported in ovarian and renal cell carcinoma (11). For the cyclin-dependent kinase inhibitor $p16$, silencing of the gene mediated by promoter hypermethylation seems to be an early event in the development and progression of the tumorigenic process in ovarian cancers. The $BRCA1$ gene promoter region is hypermethylated in a significant percentage of sporadic ovarian cancers and cell lines (10, 46). It has been reported that CpG methylation patterns are replicated with DNA replication in S phase (47), and this altered transcriptional regulation via aberrant promoter methylation plays a significant role in the tumorigenic process of ovarian cancers. Our results suggest that promoter methylation of these tumor suppressor genes plays an important and critical role in tumor development in this form of ovarian cancer. Our results have further confirmed that promoter hypermethylation of $hMLH1$ is frequently associated with MSI (7 of 19 cases, 36.8%), suggesting that a decrease or lack of $hMLH1$ expression may be responsible for mismatch repair deficiencies in this type of tumor. However, this association is still much lower than that found in other cancer types such as endometrial, colon, or gastric carcinomas with MSI, although in all of these tumor sites, MSI is not always associated with $hMLH1$ promoter methylation. Therefore, it is assumed that in GCTs, MSI may be secondary to other molecular alterations, such as somatic mutations in $hMLH1$ or $hMSH2$. It is apparent that numerous pathways are involved in maintaining genomic stability at both the nucleotide sequence and chromosomal levels, and the mutations in many genes of these pathways are likely to generate mutator phenotype. We have also confirmed that in some of these tumors, $hMLH1$ promoter hypermethylation can cause its silencing, a phenomenon that may lead to alterations in mismatch repair that represent the ultimate step in acquisition of the microsatellite mutator phenotype. Similar results have also been obtained by other investigators in sporadic carcinomas (48). Therefore, this type
of genetic instability observed in cancer cells represents intrinsic genomic instability because it involves alterations in repetitive sequences that are hot spots for mutations. We found BRCA1 promoter hypermethylation in six of eight GCTs of ovarian origin that show LOH at the BRCA1 locus on the short arm of chromosome 17. This supports Knudson’s two hits mechanism, i.e., promoter methylation on one hand, and LOH, intragenic mutations, and homozygous deletions, on the other hand, clearly demonstrated that BRCA1 also acts as a tumor suppressor gene in this type of tumor, as reported previously in different types of breast or ovarian carcinomas (49). There is another report that has linked this aberrant de novo hypermethylation of CpG islands to overexpression of the newly cloned DNMT-3 family (DNMT3A and DNMT3B). Both DNMT3A and DNMT3B are required for the establishment and maintenance of genomic methylation patterns and proper murine development (17). Both genes are up-regulated in some malignancies, including bladder, colon, kidney, and pancreatic cancers, although to varying degrees (16). Overexpression of both DNMT1 and DNMT3B is involved in human carcinogenesis, probably at different stages and through different mechanisms (50). We found that DNMT3B transcript levels were slightly increased in cancer cells compared with normal ovarian cells. Our data suggest that slightly increased expression of DNMT3B may increase the cellular potential for aberrant methylation; however, the preference for any one locus or a couple of loci to succumb to these changes may also be dictated by certain local factors. In human cancers, the end profile of methylated gene(s) will further be shaped by other events over a period of tumor progression and the selection for gene silencing events that may confer a growth and/or survival advantage. Therefore, this slight increase in DNMT3B expression in cancer cells, itself, could not explain the gene silencing, suggesting that certain other alternative mechanisms, including uncharacterized DNMTs or their altered subcellular localization in cancer cells, that could lead to global methylation errors must also be considered.

The identification of genetic instability as a primary characteristic of ovarian cancer cells suggests the development of therapeutic strategies that could target this genomic instability. It can therefore be concluded that genetic instability (both chromosomal and MSI) in sporadic ovarian cancers is relatively common than anticipated and might play an important role in the early clinical diagnosis and in chemotherapeutic management and treatment of the disease. It is likely that genes hypermethylated exclusively or more frequently in ovarian cancer than in cancers of other organ sites will be identified in the future cancers. Future studies will determine both the number of genes and which genes to be screened to obtain optimal diagnostic coverage. If our results as reported in this study are confirmed in larger studies from other laboratories (although this type of cancer is relatively rare), promoter hypermethylation, along with polymorphism and expression of DNMT3b, may have useful clinical application with regard to GCT diagnosis and management. We hope that our findings here will provide a stimulus for such future studies.

REFERENCES


The Contribution of Genetic and Epigenetic Changes in Granulosa Cell Tumors of Ovarian Origin

Varinderpal S. Dhillon, Mohammad Aslam and Syed Akhtar Husain


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/16/5537

Cited articles
This article cites 45 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/16/5537.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/10/16/5537.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.