Effect of Methyl Tetrahydrofolate Reductase (MTHFR) C766T Polymorphism on Promoter Methylation and Protein Expression of P16 Gene in Primary Ovarian Carcinoma

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Abstract: Several malignant tumors are associated with decreased expression of tumor suppressor gene P16 and one of the common causes of this suppression is epigenetic modification as methylation of promoter region; promoter methylation is triggered by enzymatic activity related to methylation reaction as MTHFR. This study was aimed to elucidate the relationship between P16 protein expression and promoter methylation in primary ovarian tumors and the liability for gene methylation in patients with mutated MTHFR. This study was conducted on 32 samples of ovarian cancer tissues and 18 samples of normal ovarian tissues were used as control group. Genomic DNA extracted from tissues was subjected to amplification with polymerase chain reaction using specific primers followed by restriction fragment polymorphism for detection of MTHFR C766T Polymorphism, after treatment with sodium bisulfite P16 promoter methylation was analyzed using methylation specific PCR (MSP). Immunohistochemical analysis was performed to examine the association of P16 methylation with protein expression. Obtained results revealed that promoter methylation of p16 was positive in 43.8% of malignant samples in contrast to 16.7 % in normal ovarian samples (p<0.05). There was significant association between promoter methylation and lack of p16 protein expression (p=0.03). Regarding MTHFR C766T Polymorphism T containing genotypes (CT+TT) constitutes 87.5% of malignant samples in comparison to 66.7% in normal samples., but there was a reverse correlation between T allele, T containing genotypes (CT+TT) was frequency and 60% of CC genotype, but no association was detected between MTHFR C766T Polymorphism and P16 protein expression or clinicopathological criteria of the tumor. Our result reflects a probable effect of MTHFR C766T Polymorphism on level of P16 promoter methylation but not on protein expression, and possibly MTHFR C766T Polymorphism and P16 promoter methylation have separate pathogenic role in ovarian cancer.


Keywords: MTHFR C7677T polymorphism, Promoter Methylation, P16 Expression, Ovarian Cancer.

1. Introduction

Tumor suppressor gene p16 (INK4A, MTS-1, or CDKN2A) located at 9p21, it is composed of 3 exons which encode 156 amino acids protein that is involved in cell cycle control (Kamb et al., 1994). P16 protein interacts with cyclin-dependent kinase CDK4 and CDK6 which bind to cyclin D regulating progression of cell from G1 to S phases. Binding of p16 to CDKs inhibits the formation of CDK/cyclin D complex, resulting in cell cycle arrest at G1 phase (Liggett and Sidransky 1998). Ablation DNA methylation is recognized as one of the most common molecular abnormalities in human cancer (Herman and Baylin 2000). This epigenetic modification occurs at the cytosines of CpG dinucleotides, which often exist in clusters called CpG islands. When methylation of these sites occurs in the promoter region of a gene, it can result in chromatin condensation and gene silencing (Issa 2003). In cancer cells, aberrant methylation frequently has been reported in tumor suppressor genes, DNA repair genes, and genes related to cancer metastasis and invasion (Esteller et al., 2001).

Among the common targets for aberrant DNA methylation is the P16 gene (Ahuja et al., 1997). The silencing of these functionally important genes leads to shift of cells from a normal cellular cycle to a state of high proliferation that favors tumor development and progression (Esteller 2005). Promoter methylation, in addition to gene deletion and point mutation of p16 locus, has been found to be one of the main mechanisms of p16 transcriptional inactivation (Huang et al., 2011). Hypermethylation of the 5’CpG islands of p16 gene promoter region has been reported to be linked to loss of protein expression in several human carcinomas including ovarian cancer (Hu et al., 2010; Malhotra et al., 2010; Shima et al., 2011).

Promoter methylation is important feature in carcinogenesis (Agrawal et al., 2007) however
the precipitating factor of aberrant DNA methylation is not completely understood. Methyleneetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) is a key enzyme in availability of methyl group that is needed for DNA methylation (Choi and Mason; 2002). It is responsible for conversion of methylene-THF to methyl-THF which is used in remethylation of sulfur-containing amino acid homocysteine to methionine. Endogenous methionine is then catabolized to produce the universal methyl donor S-adenosylmethionine (SAM) (Van der Put et al., 1998). The MTHFR gene is highly polymorphic in the general population, with the most common functional variant of 677 C to T. This polymorphism results in an alanine to valine substitution, leading to a reduction in enzyme activity [Langevin et al., 2009].

The reduction in enzyme activity impairs the folate metabolism, leading to genomic DNA hypomethylation (Ulrich et al., 2005) coexistent with gene-specific promoter hypermethylation (Oyama et al., 2004), as is frequently observed in cancer. Results on the effect of this polymorphism on cancer susceptibility are not consistent. Different types of associations have been reported in solid and hematological malignancies. The modulator role the MTHFR-677T allele has been suggested in the risk of developing human tumors as colorectal, gastric, and endometrial neoplasm and leukemia (Wiemels et al., 2001). It might be speculated therefore that MTHFR polymorphisms, and the consequent decrease in enzyme function, either protect against the development of cancer by providing more folate for DNA synthesis and repair, or increase the risk by reducing the availability of methyl groups (Friso and Choi; 2005).

So far the association of MTHFR C677T polymorphism, folate level and P16 methylation along with protein expression is hardly studied. Kamiya, et al., (1998) have advocated that folate metabolism can affect carcinogenesis through the increasing the expression of P16. Conversely, Wettergren et al., (2010) found no link between any of the MTHFR C677T polymorphic variants and P16 methylation status in colorectal cancer.

Ovarian cancer accounts for approximately 3 percent of all cancers in women and is the fifth leading cause of cancer-related death among women in the United States. It has the highest mortality of all cancers of the female reproductive system. This reflects, in part, a lack of early symptoms and proven ovarian cancer screening tests. Thus, ovarian cancer is often diagnosed at an advanced stage, after the cancer has spread beyond the ovary (Ries et al.; 2005). This indicates the need of more studies on the genetic bases and pathogenesis of ovarian cancer.

The present study was conducted to investigate the association of MTHFR C677T polymorphism with p16 promoter methylation and expression to underline the probable involvement in pathogenesis of ovarian carcinomas.

2. Materials and subjects:

Tissue samples from 50 females age range from 35 years to 70 years at diagnosis, 32 were diagnosed as primary ovarian carcinoma and 18 with normal ovary excised in patient with other gynecological lesions as multiple fibromatosis and endometrial carcinoma from pathology department in Al Baraha hospital; Dubai between 2007 and 2009 using appropriate informed consent from each patient obtained after a formal approval from institute ethics committee. Information on tumor size, histological grade and stage was abstracted from medical records. Histological types included serous cystadenocarcinoma, mucinous, endometroid, and undifferentiated carcinomas.

DNA extraction:

Tissue samples were subjected to DNA extraction by digestion with proteinase K and RNase followed by phenol/chloroform extraction and ethanol precipitation (Sambrook and Russell 2001). The methylation status of 5’ CPG islands of P16 gene was assessed by bisulfate modification of DNA and methylation specific PCR (MSP) according to the method of Herman et al. (1996). The treatment by bisulfite converts unmethylated cytosine to thymine while keeping methylated cytosine unchanged. Briefly, approximately 2 μg genomic DNA in a volume of 50 ul was denatured by 2 M NaOH at a 10:1 ratio and the sample was incubated at 37°C for 10 min. Then 30 ul of 0.1 M hydroquinone and 520 ul of 5 M Na bisulfite solution, pH 5.0, both freshly prepared were added to each sample and incubated at 50°C for 16 h. After incubation, the DNA samples were purified from the bisulfite solution using column desulphonation and elution buffer using EZ DNA Methylation™ Kit, Zymo Research. The DNA purity and concentration were determined by spectrophotometer measurement of absorbance at 260 and 280 nm.

PCR Amplification and Primers for p16 methylation:

Methylation-specific PCR (MSP) was performed on the sodium bisulfite-treated DNA samples to amplify the promoter region of the p16 gene. Two pairs of PCR primers were used in the amplification, one for methylated sequences and one for unmethylated sequences. The forward and reverse primers for the p16 unmethylated product (124 bp) were sense:
and reagents (except the primers). separately in two tubes with the same PCR conditions methylated and unmethylated products was carried out visualized under UV illumination. PCR amplification of 2% agarose gel stained with ethidium bromide and extension at 72°C. The PCR products were examined on reactions) and 1 min at 72°C followed by a final 5-min 60°C (unmethylated reactions) or 65°C (methylated 5 min, then 40 cycles consisting of 30 s at 95°C, 30 s at 58°C for 1 min and extension at 72°C. The PCR products were examined on 2% agarose gel stained with ethidium bromide and visualized under UV illumination. PCR amplification of methylated and unmethylated products was carried out separately in two tubes with the same PCR conditions and reagents (except the primers).

**MTHFR C677T Polymorphism Detection:**

For genotype analysis, the MTHFR gene was amplified in DNA extracted from all tissue samples by polymerase chain reaction followed by restriction enzyme digestion Hinf I (Promega, UK). Primer sequences, PCR conditions and restriction enzyme digestion will be as follows; (oligonucleotides were synthesized by Promega, UK).

The forward primer is 5’GGTAGTGGAAAGTTGTATTGT3’ antisense: 5’TCCCTACTCCCAACCCCA3’, for the methylated product (126 bp), the primers were p16 methylated sense 5’TGGTAGTGAAGTTGTATCGC3’, p16 methylated antisense: 5’ TCCCTACTCCCAACCCGCG3’ (antisense) (Herman et al., 1996). Amplification of the promoter region of the p16 gene was carried out using a 96-well Gene Amp PCR System 9700 thermocycler (Applied Biosystems). 50 µl PCR reaction mixture containing 2 µl of bisulfite-treated genomic DNA, dinucleotide triphosphates (dNTPs) (each at 200 µM), primers (50 pmol each per reaction), 2.5 mM MgCl2 and 1.25 U Taq in 1X PCR buffer (All reagents were supplied with the Promega). The PCR conditions were as follows: initial denaturation and hot start at 95°C for 5 min, then 40 cycles consisting of 30 s at 95°C, 30 s at 60°C (unmethylated reactions) or 65°C (methylated reactions) and 1 min at 72°C followed by a final 5-min extension at 72°C. The PCR products were examined on 2% agarose gel stained with ethidium bromide and visualized under UV illumination. PCR amplification of methylated and unmethylated products was carried out separately in two tubes with the same PCR conditions and reagents (except the primers).

Immunohistochemical analysis:

Freshly removed tissue samples were immediately fixed in 10% buffered formalin for 24 hours, embedded in paraffin, cut into sections 5-µm in thickness and mounted on slides coated with poly-L-lysine. The sections were deparaffinized by heating at 60°C for 30 min, treated with xylene, and dehydrated in alcohol. Endogenous peroxidase was blocked with 0.03% H2O2 in methanol for 20 min. The antigenic sites were unmasked by means of pressure cooker treatment for 15 min in 10 mmol/L Citrate buffer (pH 6.0). The sections were then incubated with p16 primary antibody (p16 INK4a specific monoclonal antibodies) were added for 45 min at room temperature. For detection, biotinylated horse anti-mouse or antirabbit sera (Vectastain) were applied as secondary antibodies for 30 min, followed by incubation with the avidin-biotin complex (Vectastain) for 30 min. The reaction was developed using the chromogen 3-3’ diaminodenzidine mixed with hydrogen peroxide in acetate buffer, and the sections were counterstained with hematoxylin (Malhotra et al., 2010). Ki67 immunostaining was performed to assess the proliferative activity of the tumor cells.

**Statistical analysis:**

The frequency comparisons of methylation, expression, genotype and alleles were analyzed by using Pearson chi-square or Fisher’s exact test, statistical values of P ≤0.05 were considered as significant. Odd ratios (ORs) and 95% confidence interval (CIs) were computed using the relevant 2 x 2 contingency tables for comparing p16 methylation and expression in malignant and control groups taking homozygous wild type CC genotype a reference. All statistical tests were performed using SPSS version 17 software.

3. Results:

Promoter methylation and protein expression was evaluated in 50 ovarian tissue specimens, 32 tissue samples from female patients diagnosed as ovarian cancer with mean age (53.8 ± 9.4 years), and 18 samples from females with normal ovarian tissues as control group with mean age (53.7 ± 10.9 years).

**Methylation analysis of p16 promoter:**

**As shown in figure (1)** Methylation status was evaluated in ovarian cancer and normal ovarian tissues using MSP. A band of methylated DNA was found in 37% of malignant tissues compared to 16.7% (3/18) in normal ovarian tissues, a statistical significance was found (P=0.049), patient age at diagnosis was not significantly different (P>0.05) between patients with positive methylation (mean 53.5 years) and patients without methylation (mean age 54.4 years).

**Expression of p16 protein:** (Figure 4-6).

As shown in figures (3–6) the p16 immunohistochemical staining results were
interpreted as follows: positive (+), if positive immunohistochemical staining in both nuclei and cytoplasm is present in more than 50% tumor cells; and negative (-), if positive p16 immunohistochemical staining is present in less than 10% tumor cells.

Immunostaining of the formalin-fixed, paraffin-embedded sections was reviewed by two independent observers, and strong nuclear as well as cytoplasmic staining was considered a positive reaction.

Immunohistochemical analysis was performed to evaluate difference in protein expression of p16 in tumor tissues and control group there was a significant (p value <0.05) loss of p16 protein expression in 87.5% of malignant subjects (28/32) as compared to 55.6% (10/18) in control group.

As shown in table (1): the frequency of p16 methylation, protein expression and odd ratio for ovarian cancer samples in comparison to normal control group.

**Effect of p16 promoter methylation on protein expression:**

P16 promoter methylation was matched up to protein expression and results were summarized in table (2). Our data showed that 34% (17/50) of the total analyzed samples were positive for methylation, while the lack of expression was detected in 76% (38/50) of total samples, when investigating the relation of methylation and loss of expression, we found 94.1% of methylated samples lacking protein expression and lost only in 66.7% of unmethylated samples and p value was 0.03 this correlation become non significant when we compared control and malignant groups separately as smaller number decreased statistical power.

**Genotyping of MTHFR C677T polymorphism in malignant and control groups:**

As shown in figure (2) the CC genotype gave one band of 198 bp, CT gave three bands (198bp, 175bp and 23bp), and CC gave two bands (175bp and 23bp).

As shown in table (3) no significant difference was found in between frequency of MTHFR C677T genotypes in cases with ovarian carcinoma and control group. Although not statistically significant when we pooled T allele containing genotypes (CT, and TT) in comparison to homozygous CC allele we found marked increase risk of malignancy in T containing genotypes which constitutes 87.5% (28/32) of malignant samples in comparison to 66.7% (12/18) in normal samples with odd ratio 3.5 (95%CI=0.8-14.7).

**Genotyping of MTHFR C677T polymorphism versus p16 methylation, protein expression:**

As shown in table (4) a reverse association between T allele and T containing genotype with p16 methylation, as T containing genotypes was found in 27.5% (11/40) of methylated versus 72.5% (25/40) in unmethylated samples with OR= 0.25 (CI95%=0.06-1).

Similar figure was recorded when polymorphism is correlated to methylation in malignant group only as T containing genotypes were 35.7% (10/28) methylated compared to 64.3% (18/28) unmethylated OR 0.18 (5% CI=0.02-2). Mild increase of p16 protein expression was noticed in samples with T containing genotype but with no statistical significance or risk association.

**Relationship of p16 methylation, protein expression, and MTHFR C677T polymorphism with clinicopathological parameters:**

As shown in table (5) there was no significant correlation between p16 promoter methylation, p16 protein expression, MTHFR C677T polymorphism and any of clinicopathological criteria of malignant samples. These parameters included patient’s age, tumor size, histological grade, and TNM staging. Except for mild increase risk for advanced tumor stage (3, 4) 35.7% in methylated versus 27.8% in unmethylated samples.

<table>
<thead>
<tr>
<th>Group</th>
<th>Unmethylated p16 n (%)</th>
<th>Methylated p16 n (%)</th>
<th>OR (95%CI)</th>
<th>P value</th>
<th>Negative expression n (%)</th>
<th>Positive expression n (%)</th>
<th>OR (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15/18 (83.3)</td>
<td>3/18 (16.7)</td>
<td>4.4</td>
<td>0.049</td>
<td>10/18 (55.6)</td>
<td>8/18 (44.4)</td>
<td>3.6</td>
<td>0.017*</td>
</tr>
<tr>
<td>Malignant</td>
<td>18/32 (56.2)</td>
<td>14/32 (43.8)</td>
<td></td>
<td></td>
<td>28/32 (87.5)</td>
<td>4/32 (12.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant values. P < 0.05, % = percentage values, n= number, OR= odd ratio, 95% CI= 95% confidence interval.
### Table 2: The analysis of association of p16 expression and gene methylation in ovarian tissues

<table>
<thead>
<tr>
<th>Expression (n)</th>
<th>Unmethylated p16 n (%)</th>
<th>Methylated p16 n (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples (50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>22 (66.7)</td>
<td>16 (94.1)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Positive</td>
<td>11(33.3)</td>
<td>1(5.9)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>33(100)</td>
<td>17(100)</td>
<td></td>
</tr>
<tr>
<td>Malignant group (32)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>14(77.8)</td>
<td>14(100)</td>
<td>0.08</td>
</tr>
<tr>
<td>Positive</td>
<td>4(22.2)</td>
<td>0(0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18(100)</td>
<td>14(100)</td>
<td></td>
</tr>
<tr>
<td>Control group (18)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>8(53.3)</td>
<td>2(66.7)</td>
<td>0.6</td>
</tr>
<tr>
<td>Positive</td>
<td>7(46.7)</td>
<td>1(33.3)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15(100)</td>
<td>3(100)</td>
<td></td>
</tr>
</tbody>
</table>

*Significant values. P < 0.05, percentage values are shown in parentheses, n= number.

### Table (3): Frequency of MTHFR C677T polymorphism in malignant and control groups

<table>
<thead>
<tr>
<th></th>
<th>Normal control n (%)</th>
<th>Malignant cases n (%)</th>
<th>OR 95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC genotype</td>
<td>6/18 (33.3)</td>
<td>4/32 (12.5)</td>
<td>3.5</td>
<td>0.83-14.7</td>
</tr>
<tr>
<td>CT genotype</td>
<td>11/18 (61.1)</td>
<td>25/32 (78.1)</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>TT genotype</td>
<td>1/18 (5.6)</td>
<td>3 (9.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC Genotype n (%)</td>
<td>6/18 (33.3)</td>
<td>4/32 (12.5)</td>
<td>3.5</td>
<td>0.83-14.7</td>
</tr>
<tr>
<td>CT+TT Genotypes n (%)</td>
<td>12/18 (66.7)</td>
<td>28/32 (87.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C Allele</td>
<td>23/36 (64)</td>
<td>33/64 (51)</td>
<td>1.6</td>
<td>0.7-3.8</td>
</tr>
<tr>
<td>T Allele</td>
<td>13/36 (36)</td>
<td>31/64 (48)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR= odd ratio, 95% CI= 95% confidence interval, *Significant values. P < 0.05, percentage values are shown in parentheses, n= number.

### Table (4): The MTHFR C677T polymorphism and promoter methylation and protein expression of p16 gene:

<table>
<thead>
<tr>
<th></th>
<th>CC Genotype n (%)</th>
<th>CT+TT Genotypes n (%)</th>
<th>OR 95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples</td>
<td>4/10 (40)</td>
<td>25/40 (62.5)</td>
<td>0.25</td>
<td>0.06-1.07</td>
</tr>
<tr>
<td>Methylated p16</td>
<td>6/10 (60)</td>
<td>11/40 (27.5)</td>
<td>0.18</td>
<td>0.02-2</td>
</tr>
<tr>
<td>Malignant cases</td>
<td>0/4 (0)</td>
<td>18/28 (64.3)</td>
<td>0.17</td>
<td>0.02-2</td>
</tr>
<tr>
<td>Unmethylated p16</td>
<td>4/4(100)</td>
<td>10/28(35.7)</td>
<td>0.17</td>
<td>0.02-2</td>
</tr>
<tr>
<td>Methylated p16</td>
<td>4/4(100)</td>
<td>10/28(35.7)</td>
<td>0.17</td>
<td>0.02-2</td>
</tr>
<tr>
<td>All samples</td>
<td>8/10 (80)</td>
<td>30/40 (75)</td>
<td>1.3</td>
<td>0.24-7.3</td>
</tr>
<tr>
<td>Negative expression</td>
<td>2/10 (20)</td>
<td>10/40 (25)</td>
<td>1.3</td>
<td>0.24-7.3</td>
</tr>
<tr>
<td>Positive expression</td>
<td>6/10 (60)</td>
<td>20/40 (50)</td>
<td>1.3</td>
<td>0.24-7.3</td>
</tr>
<tr>
<td>Malignant cases</td>
<td>4/4 (100)</td>
<td>24/28(85.7)</td>
<td>0.9</td>
<td>0.7-1</td>
</tr>
<tr>
<td>Negative expression</td>
<td>4/4 (100)</td>
<td>24/28(85.7)</td>
<td>0.9</td>
<td>0.7-1</td>
</tr>
<tr>
<td>Positive expression</td>
<td>0/4 (0)</td>
<td>4/28(14.3)</td>
<td>0.9</td>
<td>0.7-1</td>
</tr>
</tbody>
</table>

OR= odd ratio, 95% CI= 95% confidence interval, p <0.05 = statistical significance.
Table (5): Clinicopathological criteria of ovarian cancer patients in relation to promoter methylation and protein expression of p16 gene and MTHFR C677T polymorphism:

<table>
<thead>
<tr>
<th>Parameter (n)</th>
<th>Unmethylated p16 n (%)</th>
<th>Methylated p16 n (%)</th>
<th>OR (95% CI)</th>
<th>P value</th>
<th>Negative expression n (%)</th>
<th>Positive expression n (%)</th>
<th>OR (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade: Low(18)</td>
<td>10 (71.4) 4 (28.6)</td>
<td>8 (44.4) 10 (55.6)</td>
<td>0.32 (0.07-1.4)</td>
<td>0.12</td>
<td>2 (50) 16 (57.1)</td>
<td>0 (0) 12 (42.9)</td>
<td>1.3 (0.17-10.8)</td>
<td>0.6</td>
</tr>
<tr>
<td>High(14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 (50) 16 (57.1)</td>
<td>2 (50) 12 (42.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage Early(22)</td>
<td>9 (64.3) 5 (35.7)</td>
<td>13 (72.2) 5 (27.8)</td>
<td>0.7 (0.15-3.11)</td>
<td>0.7</td>
<td>4 (100) 0 (0)</td>
<td>18 (64.3) 10 (53.7)</td>
<td>0.64 (0.5-0.9)</td>
<td>0.2</td>
</tr>
<tr>
<td>late(10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 (50) 20 (71.4)</td>
<td>2 (50) 8 (28.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Small(20)</td>
<td>10 (71.4) 4 (28.6)</td>
<td>10 (55.6) 8 (44.4)</td>
<td>0.5 (0.11-2.2)</td>
<td>0.29</td>
<td>2 (50) 18 (64.3)</td>
<td>2 (50) 10 (35.7)</td>
<td>0.6 (0.07-4.5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Large(12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 (50) 12 (42.9)</td>
<td>2 (50) 12 (42.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt; 45(7) Mean ± SD</td>
<td>3 (21.4) 11 (78.6) 53.5 ± 9.3</td>
<td>4 (22.2) 14 (78.8) 54.4 ± 11.3</td>
<td>1.04 (0.19-5.69)</td>
<td>0.65</td>
<td>3 (25) 11 (75) 6 (21.4) 22 (78.6)</td>
<td>0.8 (0.07-9.4)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>&gt; 45(25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 (14.3) 3 (12)</td>
<td>6 (85.7) 22 (88)</td>
<td>0.8 (0.07-9.3)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Histological types:
- Serous (14): 10 (71.4) 4 (28.6)
- Mucinous (6): 4 (66.7) 2 (33.3)
- Endometroid (4): 2 (50) 2 (50)
- Undifferentiated (8): 4 (50) 4 (50)

OR= odd ratio, 95% CI= 95% confidence interval, p <0.05 = statistical significance.

Figure 1: methylation specific polymerase chain reaction of p16 gene in ovarian cancer and normal tissues:

M: polymerase chain reaction PCR with primers for methylated p16 gene, showing positive 126 bp bands in lanes 2,3,6,8,10. U: polymerase chain reaction PCR repeated for the same samples with primers for unmethylated p16 gene, showing positive 124 bp bands in lanes 4,5,7,9,11, lane I in both gels is 50 bp DNA marker.

Figure (2): Agarose Gel electrophoresis for MTHFR C677T polymorphism:

Lane 1 (DNA marker), lane 2,4,5 (TT ) genotype. Lane 3 and 6 (CC) genotype. Lane 7 and 8 (CT) genotype.
4. Discussion:

Ovarian cancer is still considered one of the most fatal gynecologic malignancies (Ozols et al., 2005). According to American cancer society (2011), it was estimated that 21,990 women would develop ovarian cancer, and 15,460 women would die from the disease in the United States.

In spite of the progress in cancer research and treatment, no satisfactory improvement in survival for patients with ovarian cancer remains low; as more than 50% of patients die within 5 years of their ovarian cancer diagnosis (McGuire et al., 1996).

Disruption of the normal DNA methylation patterns is a recognized common feature of human cancer cells (Esteller and Herman; 2002). It has been observed that promoter methylation of specific genes in carcinomas occur in both a tissue specific and cell specific manner making identification of methylation patterns a potentially useful tool for cancer management. (Costello et al., 2000) This may be especially important for patients with ovarian cancer, because early detection of the disease can improve survival; there is a large difference in 5-year survival between patients with localized, Stage I tumors (94%) and patients with Stage III or IV disease (29%) (Jemal et al., 2005).

The aberrant methylation of the CpG island located in the 5′-promoter region of several tumor suppressor genes such as hMLH1, BRCA1, VHL, CDH1, p16\(^{INK4a}\), and APC shuts down the expression of these contiguous genes (Baylin et al., 2001; and Esteller & Herman 2002). P16 is a tumor suppressor gene which has an important role in cell cycle through inhibiting binding of CDK4 to cyclin D1 (Merlo et al., 1995). Investigating association of p16 methylation and expression is still in focus of
interest in carcinogenesis and prognosis of human malignancy.

In the current study using MSP there was significant increase in frequency of promoter methylation of p16 in malignant ovarian tissue (43.8 %) as compared to normal control group (16.7%). Similar results were also reported by other investigators as Andrew et al., 2006 who recorded positive methylation in 41.6% of malignancy and 21.1% in borderline ovarian tumor. Another study on 249 patients with ovarian cancer was performed by Katsaros et al., (2004) who found 40% positive in malignant tissues. But previous results using PCR-RFLP which is less sensitive than MSP showed less frequent as McCluskey et al., (1999) who found 5% of methylation, and 33% methylation was reported by Li et al., (2006) in ovarian cancer tissues.

Increased p16 promoter methylation has been identified to be a ubiquitous mechanism of gene silencing that play a significant role in tumorigenesis of several human cancers not only ovarian including oral squamous cell carcinoma (Kaur et al., 2010), hepatocellular carcinoma (Fukai et al., 2005), colon (Malhotra et al. 2010), breast (Nielsen et al., 2001), gastric (Hu et al., 2010), and cervical carcinomas (Huang et al., 2011).

The mechanism of p16 methylation linked carcinogenesis is mostly transcriptional silencing of gene expression (Kriege et al., 2011). Clinical studies indicated that p16 expression is undetectable in about a third of ovarian cancer cases (Sui et al., 2000; and Havrilesky et al., 2001), and that patients with low p16 expression have poor response to chemotherapy and unfavorable survival outcome (Kudoh et al., 2002). Cell culture experiments demonstrate that reintroducing functional p16 into p16-null ovarian cancer cells results in inhibition of cell growth and increases in apoptosis, suggesting that p16 plays a role in ovarian cancer progression (Wolf et al., 1999). Li et al., (2006) found significant reduction of expression in ovarian cancer than normal tissues and lack of expression was mainly in methylated samples.

In the present study we used immunohistochemistry to evaluate p16 protein expression, our results detected loss of expression in 87.5% (28/32) of malignant samples, while in normal samples it was 55.6% (10/18), and there was significant correlation between promoter methylation and loss of protein expression as 94% (16/17) of total methylated samples (normal and malignant) showed loss of p16 protein expression, while it was found in 66% (22/33) of unmethylated samples, in malignant samples loss of expression was found in 100% (14/14) of methylated and 77.8% (14/18) of unmethylated samples, this indicates that methylation increase p16 inactivation but it is not the only reason, it may be a result of other mechanism exhibited by previous works such as transcriptional errors (Chen et al., 1997) or posttranslational mechanisms as gene deletion or mutation (Fujita et al., 1997).

Numerous previous studies investigated the relationship of inactivation or epigenetic changes of p16 gene with prognosis of ovarian tumors and other human tumors. Kudoh et al., (2002) found that ovarian cancer with low expression didn’t respond well to chemotherapy, and were associated with poor prognosis, similar findings were reported by Andrew et al., (2006); but they found significant increase risk of disease progression and overall survival but this was not available in our study. Moreover the prognostic value of p16 methylation was proved in many malignant tumors to be an indicator of poor prognosis and/or biomarker for response to chemotherapy (Goto et al., 2010; Wettergren et al., 2008; Csepregi et al., 2010; and Endo et al., 2011). Kaur et al., (2010) found significant association of p16 methylation and nodal involvement in oral squamous cell carcinoma. Decrease expression of p16 protein was significantly correlated with large tumor size in leiomyosarcoma (Kawaguchi et al., 2003). In our study no correlation was found between p16 methylation, expression, and various clinicopathological factors including age, tumor stage, and grade. This disagreement may be caused by the heterogenous nature of ovarian tumors and small size of investigated samples.

Limited number of studies investigated the association of MTHFR C677T and ovarian cancer with controversial results (Terry et al., 2010; and Magnowski et al., 2010). In the current study we recorded more frequency of T allele and T containing genotypes (CT+TT) in ovarian cancer tissues (48%, 87.5%) than in normal ovarian tissues (36%, 66.7%) respectively with OR 1.6 (0.7-3.8) for T alleles and OR 3.5 (0.8-14.7) for (CT+TT) genotype showing the potential conflict of MTHFR C677T mutation on ovarian carcinogenesis. This may be explained by the fact that among 677TT (Val/Val) individuals, the MTHFR enzyme is less efficient in converting 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, thus potentially preventing depletion of 5, 10-methylenetetrahydrofolate, a cofactor for de novo DNA synthesis, especially dTMP. As a result, cells may be less prone to "dNTP stress" which has been shown to promote cancer-associated genetic alterations due to alterations in the pool of nucleotide precursors available for DNA synthesis. Alteration of these precursor pools induced by methyl (folate) deficiency significantly increases the uracil content
and the frequency of chromosome breakages in human leukocyte DNA (Deloughery et al., 1996).

MTHFR C677T genotype has been analyzed in relation to promoter methylation of p16 in tumors from different sites with controversial results, Tao et al., (2009) found no relation to p16 promoter methylation in breast cancer; Curtin et al., (2007) found an increased likelihood of highly CpG-methylated phenotype in colon tumors for those with one or two variant MTHFR 1298C alleles, and the association was modified by high-risk dietary profiles (low folate and methionine intake and high alcohol use). Similarly, MTHFR C677T, A1298C, or MTR C2756G genotypes were not associated with E-cadherin and p16 promoter methylation in esophageal (Wang et al., 2008) and cervical cancers (Kang et al., 2005). A pooled analysis including 725 cases esophageal carcinoma and 1531 controls showed a significant association between the MTHFR 677 TT genotype and susceptibility to esophageal cancer [Langevin et al., 2009]. Lu et al., (2011) found that diet folate intake had different effects on the prognosis of esophageal carcinoma by different genotypes of MTHFR C677T. The preventive effect of folate intake was more evident in patients carrying MTHFR 677CC genotype.

In our research we analyzed MTHFR C677T polymorphism and compared its frequency to p16 methylation and expression in ovarian cancer. we demonstrated marked decrease of p16 promoter methylation in CT+ TT genotypes than CC genotype in all studied individuals, and in separate malignant and control groups with OR 0.25 (0.06-1.07), and 0.18 (0.02-2) respectively with no significant difference between malignant and control group regarding decline of p16 methylation (data not shown). This coincide with finding of Chiusolo et al., (2006) in multiple myeloma who demonstrated that MTHFR 677CC is associated with a higher prevalence of p16 hypermethylation. In our study no clear association was found between polymorphism and p16 protein expression or any of clinicopathological criteria of ovarian carcinomas.

We conclude that p16 methylation is an important carcinogenic factor on ovarian tissue through inhibition of gene expression, and there is potential effect of T allele and T containing genotype of MTHFR C677T polymorphism on degree of methylation of p16 gene, as MTHFR C677T polymorphism was associated with reduction in P16 promoter methylation and increased expression of p16 gene, meanwhile it is linked to increased risk of ovarian cancer so mostly carcinogenic effect is not through modifying the methylation of p16. However a remarkable risk of ovarian carcinogenesis was noticed in separate analysis of the effect of MTHFR mutation and p16 methylation on ovarian tissues. This may reflect the possible interaction of other genetic, environmental and dietary factors that can affect DNA expression and epigenetic changes that needs to be further declared.

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