Methylation of SFRPs and APC Genes in Ovarian Cancer Infected with High Risk Human Papillomavirus

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Abstract

Background: Secreted frizzled-related protein (SFRP) genes, new tumor suppressor genes, are negative regulators of the Wnt pathway whose alteration is associated with various tumors. In ovarian cancer, SFRPs genes promoter methylation can lead to gene inactivation. This study investigated mechanisms of SFRP and adenomatous polyposis coli (APC) genes silencing in ovarian cancer infected with high risk human papillomavirus.

Materials and Methods: DNA was extracted from 200 formalin-fixed paraffin-embedded ovarian cancer and their normal adjacent tissues (NAT) and DNA methylation was detected by methylation specific PCR (MSP). High risk human papillomavirus (HPV) was detected by nested PCR with consensus primers to amplify a broad spectrum of HPV genotypes.

Results: The percentages of SFRP and APC genes with methylation were significantly higher in ovarian cancer tissues infected with high risk HPV compared to NAT. The methylated studied genes were associated with suppression in their gene expression.

Conclusion: This finding highlights the possible role of the high risk HPV virus in ovarian carcinogenesis or in facilitating cancer progression by suppression of SFRP and APC genes via DNA methylation.

Keywords: Ovarian cancer - HPV - DNA methylation - SFRPs and APC genes - MSP - Wnt pathway

Introduction

Ovarian cancer is a lethal tumor of female genital tract (Siegel et al., 2012). Its incidence is high in developed countries, with rates exceeding 9/100,000 women per year with 5-year survival rate of 15-20% due to chemoresistance (Ozdemir et al., 2012). In Saudi Arabia, ovarian cancer ranks the seventh among females and accounts for 3.1% of newly diagnosed cases with median age of 50 years (Al-Eid, 2007). Human papillomavirus (HPV) is considered as one of the environmental factors causing ovarian cancer worldwide (Malisic et al., 2012; Shanmughapriya et al., 2012). E6 and E7 genes of HPV (HPV) was detected by nested PCR with consensus primers to amplify a broad spectrum of HPV genotypes. Results: The percentages of SFRP and APC genes with methylation were significantly higher in ovarian cancer tissues infected with high risk HPV compared to NAT. The methylated studied genes were associated with suppression in their gene expression. Conclusion: This finding highlights the possible role of the high risk HPV virus in ovarian carcinogenesis or in facilitating cancer progression by suppression of SFRP and APC genes via DNA methylation.

Keywords: Ovarian cancer - HPV - DNA methylation - SFRPs and APC genes - MSP - Wnt pathway

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(APC) gene is identified in germ line of individuals with familial adenomatous polyposis coli (Chen et al., 2011; Fang et al., 2011).

The silencing of SFRP genes leads to the oncogenic activation of Wnt pathway and contributes to ovarian cancer progression. This study investigated the mechanism for SFRPs and adenomatous polyposis coli (APC) genes loss in ovarian cancer infected with high risk human papillomavirus.

Materials and Methods

This study was conducted in compliance with Helsinki Declaration 2013 and was approved by the ethical committee of the college of medicine, King Saud University. Two hundred formalin-fixed paraffin-embedded (FFPE) ovarian cancer and their normal adjacent tissues (NAT) were collected from the pathology department, college of medicine, King Saud University and the Pathology Department, Riyadh Regional Laboratory and blood bank, Saudi Arabia.

The patients’ mean age was 48±11.9 years (range 23-85 years). The histological stages were determined according to the International Federation of Gynecology and Obstetrics system (FIGO). Ovarian cancer samples were classified as followed: 18% had stage-I, 33.5% stage-II, 31.5% stage-III and 17% stage-VI.

DNA extraction and bisulfate treatment

All FFPE samples were thin sectioned at 8μm thickness using Microtome (Leica, Manual Rotary Microtome RM2235). Tissue sections were floated on DEPC-treated water bath then were picked up on glass slides and were allowed to air dry. For DNA extraction, two tissue sections were examined and tumor area tissues were removed using scalpel. Nucleic acids were extracted using Recover All total Nucleic Acid Isolation Kit (Ambion, Life Technologies, USA) following the manufacturer’s instructions. The quantity and quality of the extracted DNA was described by using NanoDrop 8000 spectrophotometer (Thermo scientific, USA).

The genomic DNA was treated with sodium bisulfate using EpiTect Bisulfite Kits (QIAGEN, Germany) according to the manufacturer’s instructions. This process converts the non-methylated cytosine residues to uracil, while the methylated cytosine unchanged. Briefly, 2μg of the extracted DNA was incubated with 140μl of EpiTect Bisulfite reaction mixture at room temperature for 3min followed by 99°C for 5min, 60°C for 25min, 99°C for 5min, 60°C for 85min, 99°C for 5min and finally 60°C for 175min. The BL buffer containing 10μg/ml carrier RNA was mixed with bisulfite converted DNA and was transferred to the EpiTect spin columns followed by washing then elution steps.

Methylation-specific PCR (MSP):

The CpG islands of SFRP1, SFRP2, SFRP4, SFRP5 and APC genes were examined by MSP (TAKARA, BIO INC, Japan). The forward and reverse primers corresponding to the predicted sequence of methylated or unmethylated genes used in this study were shown in Table 1 as previously described (Urakami et al., 2006; Perri et al., 2007; Veeck et al., 2012). For the reaction, 200ng of sodium bisulfate treated DNA was added to 25μl of 2X reaction buffer containing, 0.3μM of each forward and reverse primers, 1.2μl of MSP enzyme, 0.5μl of 100X SYBR Green I and 1μl of 50X of ROX reference Dye. The reaction was done in the ABI 96-Well Optical Reaction Plate. The amplification conditions were 95°C for 5min followed by 40 cycles of 30 sec denaturation at 98°C, 30 sec annealing at 55°C and 45 sec extension at 72°C. All reactions were performed using an ABI 7500 System (Applied Biosystem, life technology, USA). Polymerase chain reactions were performed in triplicates for all samples. Following the amplification reaction, the melting curve analysis was performed to analyze the reaction specificity. MSP products were separated electrophoretically on 3% agarose and band of methylated and/or unmethylated genes were visualized by photo-documentation system (syngene bio imaging, USA) (Figure1).

The detection of human papillomavirus (HPV) by nested PCR:

Nested PCR with consensus primers MY09/MY11 was used to amplify a wide spectrum of HPV types with PCR product of 450bp followed by using primers GP5+/GP6+ with PCR product of approximately 150bp. Each sample was tested triplicates. The primers sequences were shown in Table 1 as previously described (Qu et al., 1997). The PCR reaction was done in 50μl, contained 500ng of DNA, 1X PCR Master Mix (Promega, Madison, USA), 3mM MgCl₂, 200μM dNTPs, 300nM of each primer. Amplifications using MY09/MY11 were performed with the following cycling profile: 94°C for 5min followed by 40 cycles of 1min denaturation at 95°C, 1min annealing at 55°C, and 1min elongation at 72°C. The last cycle was followed by a final extension of 10min at 72°C. The primer annealing step of GP5+/GP6+ primers-based PCR was performed at 40°C for 2min. During amplification positive and negative control samples were included. PCR products were analyzed on a 2% agarose gel and visualized by UV-trans illuminator.

Identification of HPV genotyping by direct DNA sequencing

To identify the genotypes of HPV, all positive PCR products were analyzed by direct DNA sequencing according to our recent study (Al-Shabanah et al., 2013). Chromatograms with sharp peaks and quality values ≥20

![Image](https://via.placeholder.com/150)
with little or no background noise consider as single HPV infection. The nucleotide sequences were subsequently subjected to Basic Local Alignment Search (BLAST).

Total RNA extraction, cDNA synthesis and Real time PCR:

Total RNA was extracted from FFPEs using RecoverAll Nucleic Acid Isolation Kit (Ambion, 1975, life technology, USA) following the manufacturer instructions. The quantity of extracted RNA was characterized using Nanodrop 8000 in which all the isolated RNA samples had 260/280 ratio of 1.9-2.1.

cDNA was prepared from 1µg RNA using high capacity cDNA reverse transcriptase kit Applied biosystem according to manufacturer’s instructions (Applied biosystem, life Technology, CA, USA). The quality of cDNA was confirmed by amplifying GAPDH gene. PCRs were carried out using SYBR Green PCR Master Mix Reagents Kit (Applied biosystem, life Technology, USA). The expression levels of the studied genes were expressed as relative expression using ∆∆Ct method. The cycling program was 5min at 95ºC; 40 cycles of 30 sec at 95ºC denaturation, 1 min at 60ºC annealing and extension followed by melting curve. Primer sequences used in this study were shown in Table 1 as previously described (Cheng et al., 2007; Rigi-Ladiz et al., 2011).

Statistically analysis:

Methylation percentages across genes and tumor characteristics (e.g. age, tumor stage, HPV infection and its genotyping) were analyzed using exact chi-square test (SPSS, version 17.0). p values<0.05 were considered statistically significant.

Results

The percentages of methylation in the cancer and normal adjacent tissues

The percentage of APC, SFRP1, SFRP2, SFRP4 and SFRP5 genes DNA methylation were investigated in ovarian malignant and their normal adjacent tissues using methylation-specific PCR analysis. In ovarian cancer tissues, the percentages of SFRP1, SFRP2, SFRP4 and SFRP5 genes methylation were 42%, 63%, 51% and 55% compared to 3%, 5%, 4% and 2% in NAT (p<0.05) respectively. APC gene methylation was 36% in ovarian cancer compared to 10% in NAT (p<0.05) (Figure 2).

The association between the clinico-pathological data and the gene methylation

The clinico-pathological data of the methylated genes are summarized in Table 2. In the age group <40 years, SFRP1,-2,-4,-5 and APC genes methylation were 49.1%, 54.5%, 60%, 54.5% and 32.7% compared to 39.3%, 66.2%, 47.6%, 55.2% and 37.2% in age group >40 years respectively. In relation to age group there was significant difference in SFRP2 gene in age >40 years compared to <40 years old Figure 3.

Of tumor samples, 18% had stage-I, 33.5% stage-II, 31.5 % stage-III and 17 % stage-VI histological types. The percentage of SFRP1 gene methylation was significantly

Figure 2. The Percentage of Genes Methylation in Ovarian Cancer Compared to Their Normal Adjacent Tissues

Figure 3. The Percentage of Gene Methylation in Ovarian Cancer Tissues in Relation to age Group

Figure 4. The Percentage of Gene Suppression in Methylated and Un-Methylated Groups
Table 2. Clinicopathological Data Age Histological Stage, HPV Infection and Genotyping with Methylated Genes

<table>
<thead>
<tr>
<th>Variable total=200</th>
<th>SFRP1</th>
<th>SFRP2</th>
<th>SFRP4</th>
<th>SFRP5</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age&lt;40 (n=55)</td>
<td>27 (49.1%)</td>
<td>30 (54.5%)</td>
<td>33 (60%)</td>
<td>30 (54.5%)</td>
<td>18 (32.7%)</td>
</tr>
<tr>
<td>&gt;40 (n=145)</td>
<td>57 (39.3%)</td>
<td>96 (66.2%)</td>
<td>69 (47.6%)</td>
<td>80 (55.2%)</td>
<td>54 (37.2%)</td>
</tr>
<tr>
<td></td>
<td>p=0.13</td>
<td>p=0.08</td>
<td>p=0.18</td>
<td>p=1.0</td>
<td>p=0.6</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIGO I (n=36)</td>
<td>8 (22.2%)</td>
<td>24 (65.7%)</td>
<td>20 (55.6%)</td>
<td>18 (50%)</td>
<td>7 (19.4%)</td>
</tr>
<tr>
<td>FIGO II (n=67)</td>
<td>29 (43.3%)</td>
<td>36 (53.7%)</td>
<td>33 (49.3%)</td>
<td>35 (52.2%)</td>
<td>21 (31.3%)</td>
</tr>
<tr>
<td>FIGO III (n=63)</td>
<td>29 (46%)</td>
<td>44 (69.8%)</td>
<td>28 (44.4%)</td>
<td>35 (55.6%)</td>
<td>26 (41.3%)</td>
</tr>
<tr>
<td>FIGO VI (n=34)</td>
<td>18 (52.9%)</td>
<td>22 (64.7%)</td>
<td>21 (61.8%)</td>
<td>22 (64.7%)</td>
<td>18 (52.9%)</td>
</tr>
<tr>
<td>HPV infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV+ (n=72)</td>
<td>47 (65.3%)</td>
<td>44 (61.1%)</td>
<td>39 (54.2%)</td>
<td>45 (62.5%)</td>
<td>45 (62.5%)</td>
</tr>
<tr>
<td>HPV- (n=128)</td>
<td>37 (28.6%)</td>
<td>82 (64.1%)</td>
<td>63 (49.2%)</td>
<td>65 (50.8%)</td>
<td>p=0.07</td>
</tr>
<tr>
<td>HPV+* (n=36)</td>
<td>29 (80.5%)</td>
<td>23 (63.9%)</td>
<td>23 (63.9%)</td>
<td>25 (69.4%)</td>
<td>24 (66.6%)</td>
</tr>
<tr>
<td>HPV+* (n=47)</td>
<td>29 (61.7%)</td>
<td>28 (59.6%)</td>
<td>25 (53.2%)</td>
<td>27 (57.4%)</td>
<td>27 (57.4%)</td>
</tr>
<tr>
<td>HPV+* (n=18)</td>
<td>12 (66.7%)</td>
<td>10 (55.6%)</td>
<td>8 (44.4%)</td>
<td>13 (72.2%)</td>
<td>p=0.02</td>
</tr>
</tbody>
</table>

*indicates infection with genotype alone or mixed infection with other genotype

higher in stage VI (52.9%) than in other stages. Also it was significantly high in stage-II 43.3% and stage-III 46% than in stage-I 22.2%. SFRP-2,-4,-5 genes methylation were statistically insignificant in relation to tumor stage as in Table 2. The APC gene methylation was significantly increase with the tumor stage increasing in which 52.9% was methylated in stage-VI compared to 19.4%, 31.3% and 41.3% in stage-I, -II and -III respectively.

The DNA methylated genes percentages were higher in HPV positive samples than in negative one. The percentages of SFRP-1, SFRP-5 and APC gene methylation were significantly high in HPV positive 65.3%, 62.5% and 62.5 compare to the negative cases 28.6%, 50.8% and 21% respectively. In HPV positive samples, SFRP-2 and-4 genes methylation was 61.1% and 54.2% compared to 64.1% and 49.2% in negative group respectively (Table 2).

The association between HPV infection/genotyping with gene methylation

The percentages of HPV genotypes distribution and gene methylation in cancer samples were shown in Table 2. Among the 200 studied samples, the common HPV genotypes alone or mixed with other genotypes were 23.5% HPV-16, 18% HPV-18 followed by 9% HPV-45. The infection with one genotype was 23 cases HPV-16, 20 cases HPV-18 and 3 cases HPV-45. The mixed infection with HPV-16/18 was 11 cases, HPV 16/45 was 10 cases, HPV-18/45 was 2 cases or HPV=16/18/45 was 3 cases.

In ovarian cancer samples, the percentages of genes methylation differ with different HPV genotype. In HPV-16 genotype alone, high percentages of SFRP-2, SFRP4 and SFRP5 genes methylation were observed in 61%, 52% and 57% respectively. Also in HPV-18 alone, high percentages of SFRP-1,-2,-4,-5 and APC genes methylation were observed in 70%, 65%, 55%, 75% and 65% respectively. Similarly, high percentages of SFRPs and APC were observed in mixed infection as shown in Table 3.

The correlation between DNA methylation with the gene expression levels

There was a significant correlation between the expression levels of SFRP1, SFRP2, SFRP4, SFRP5 and APC genes and their methylation in cancer tissues. SFRP1 gene expression level was down-regulated in 97% of the methylated SFRP1 group and in 3.4% of the unmethylated group. SFRP2 gene was down-regulated in 80.2% of the methylated SFRP2 group and in 17.6% of the unmethylated group. SFRP4 gene expression was down-regulated in 89.2% of the methylated SFRP4 group and in 17.3% of unmethylated group. SFRP5 gene was down-regulated in 79.1% of the methylated SFRP5 group and in 22.2% of the unmethylated group. APC gene was down-regulated in 75% of the methylated APC group and in 15.6% of the unmethylated group as in Figure 4.

Discussion

Ovarian tumor is a common neoplasm of female genital tract and a lethal gynecologic malignancies (Lengyel, 2010). The aberration of some genes in cancer may be used as specific markers during carcinogenesis process. This study focused on the DNA methylation and expression of APC and SFRPs genes in ovarian cancer in relation to HPV infection. Wnt signaling pathway plays an important role in the development, proliferation, differentiation and apoptosis of cells (Kawano et al., 2003). The DNA methylation and expression of SFRP genes are important in regulating or blocking Wnt signaling pathway (Su et al., 2010). Several studies showed methylation in the Wnt signaling-related molecules in cancer (Shen et al., 2011) with controversy in ovarian cancer (Boy et al., 2010; Bernard et al., 2012; Usongo et al., 2013). The CpG island methylation, together with the down-regulation of SFRPs genes, was reported in several cancers, such as colorectal cancer, ovarian cancer, breast cancer and gastric cancer.
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The aberrant promoter methylation of the genes may result from mis-targeted host defense methylation during viral integration or as a consequence of genomic instability due to HPV infection (Marsit et al., 2006). In conclusion,

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this study showed that in the high risk HPV infected patients, the DNA methylation is one of the mechanisms that may lead to SFRPs and APC genes loss in ovarian cancer. The study of HPV viral infection in ovarian cancer with relation to SFRP1, -2, -4, -5 and APC genes may have best predictive values in detecting individuals at risk for cancer.

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References


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