Original Article

Investigating RASSF1A hypermethylation and endometrial carcinoma occurrence and development using in vivo and in vitro demethylation of 5-AZA-2’-deoxy cytidine

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Abstract: Objective: To explore the role of RASSF1A in the occurrence and development of endometrial cancer. Methods: In vitro experiments used human endometrial cancer cells (HEC-1-B) treated with different concentrations of 5-AZA-2'-deoxy cytidine (5-Aza-CdR). In vivo experiments used HEC-1-B-treated mice that were exposed to combinations of cisplatin (DDP), medroxyprogesterone acetate (MCP), and/or 5-Aza-CdR. Analysis was then performed using immunohistochemical, MTT, methylation-specific PCR, fluorescence quantitative PCR, Western blotting, and TUNEL staining approaches. Results: When compared to both atypical hyperplasia and normal endometrial tissue, RASSF1A protein expression was significantly reduced in endometrial carcinoma tissues (P<0.01). There was no significant difference between atypical or normal endometrial tissue (P = 0.692). There were no significant differences in RASSF1A protein expression in endometrial carcinoma across age (P = 0.418), tumor grade (P = 0.433), stage (P = 0.873), or classification (P = 0.520). Furthermore, RASSF1A promoter regions were abnormally hypermethylated, leading to decreased mRNA and protein expression in HEC-1-B cells and xenograft tissues. 5-Aza-CdR application reversed the abnormal RASSF1A hypermethylation, inhibited transplanted tumor growth, and induced apoptosis in a concentration-dependent manner. Finally, 5-Aza-CdR co-application significantly strengthened the efficacy of both DDP and MPA in tumor suppression. Conclusions: Either inactivation or low expression of RASSF1A may play a role in the occurrence of endometrial cancer, but the determining factor may be the abnormal hypermethylation of RASSF1A. RASSF1A is a potential target, making demethylation a possible, effective treatment option for endometrial cancer.

Keywords: Demethylation, DNA methylation, endometrial neoplasms, epigenetics, RASSF1A

Introduction

The Ras-association domain family 1A (RASSF1A) gene was first described as a tumor suppressor gene by Damman et al. [1] in 2000. Although RASSF1A is expressed in all normal tissues, it is either decreased or deleted in more than half of tumors and tumor cell lines. This decrease or deletion occurs in a wide variety of cancers, including lung cancer [2], breast cancer [3, 4], hepatocellular carcinoma [5], bladder [6], colorectal [7], and kidney [8], among others. To this end, hypermethylation of the CpG island in the RASSF1A promoter region is widely found in tumors [9-11]. Demethylation can also result in induced or exogenous expression of RASSF1A, leading to tumor growth inhibition [12-15]. At present, there are two main mechanistic theories regarding RASSF1A functioning as a tumor suppressor gene. First, RASSF1A transcription regulates proliferation in the G1/S phase of the cell cycle [16]. Second, RASSF1A is a negative regulator in the Ras activation signaling pathway, meaning that RASSF1A protein expression plays a role in the anti-cancer response by blocking the
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cell cycle, promoting apoptosis, and resisting microtubule depolymerization [17]. Consequently, absence or inactivation of the gene can destroy the balance of Ras signaling and lead to malignant cellular transformation.

DNA methylation is a common epigenetic molecular mechanism that involves reversible chemical modification. This occurs when the enzyme DNA methyltransferase (DNMT) adds a methyl group onto a cytosine base [18]. In contrast, demethylation refers to any mechanisms that can reverse or inhibit this methylation [18, 19]. The demethylation drug 5-Aza-CdR is a nucleoside DNMT inhibitor [20, 21], which can be phosphorylated by deoxycytidine kinase in tumor cells. This phosphorylation leads to DNA incorporation as a phosphate during the process of DNA replication. 5-Aza-CdR can inhibit DNA synthesis, induce cell death at high concentration, and exert cytotoxic effects at the low. Furthermore, it can replace unmodified tumor cell cytosines, rendering DNMT inactive without inducing cell death [22-24]. DNA methylation has been correlated with gene expression; demethylation in particular, has been shown to restore the expression of multiple tumor suppressor genes [20, 25, 26]. 5-Aza-CdR is the most widely studied DNMT inhibitor and has been approved by the FDA for the treatment of some malignant tumors [27].

Despite this, little research has been conducted regarding the relationship between RASSF1A gene expression and endometrial cancer. As such, we sought to examine the role RASSF1A played in the occurrence and development of endometrial cancer. Secondarily, we sought to explore the clinical applications of 5-Aza-CdR in endometrial carcinoma treatment, utilizing different diverse systems and levels of analysis.

Materials and methods

Ethics statement

The study was approved by the Human Investigation Ethics Committee of West China Second Hospital, an affiliate of Sichuan University. All endometrial carcinoma samples were only collected after written, informed consent was obtained from all patients. All animal experimental protocols were approved by the Chinese Institutional Ethics Review Committee for Animal Experimentation.

Tissue specimens

Tissue samples for immunohistochemical experiments were obtained from West China Second University Hospital between 2004 and 2007. Tumor stage and histological grade were established according to the criteria set forth by the Federation of International Gynecology and Obstetrics (FIGO, 2000) surgical staging system. None of the patients underwent hormone therapy, radiotherapy, or chemotherapy prior to surgery.

Cell culture and treatment groups

The HEC-1-B human cell line is a moderately differentiated, endometrial adenocarcinoma cell line. HEC-1-B cells were obtained from the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, China). According to the provider’s instructions, cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C in a humidified atmosphere containing 5% CO2. HEC-1-B cells were randomly divided into control and experimental groups and inoculated in 96-well plates at a concentration of 1 × 105/mL, 200 µl/well. Next, cells were cultivated with serum-free growth media after 24 h and treated with different concentrations of 5-Aza-CdR(AZA): group A (blank control group, AZA 0 mol/L), group B (AZA 1.0 × 10-6 mol/L), group C (AZA 3.0 × 10-6 mol/L), and group D (AZA 10 × 10-6/L). Length of drug effects were examined using a variety of methods, which revealed differential time courses. Methylation specific PCR (MSP) and real-time quantitative PCR revealed drug effects lasting for 24 h. In contrast, Western blotting showed detectable effects up to 48 h and TUNEL staining showed drug effects up to 72 h.

Immunohistochemistry

Immunohistochemical experiments were conducted using a commercially available streptavidin-biotin complex (SABC) staining kit (Boster, USA) and according to the manufacturer’s instructions. Briefly, tissue sections were de-waxed, hydrated, and endogenous peroxidase activity quenched using hydrogen peroxide. Antigen retrieval was induced using heat, after which tissue was blocked usingBSA. After blocking, a RASSF1A polyclonal antibody (1:50, Santa CruzBiotechnology, USA) was incubated
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overnight with the tissue at 4°C. Sections were then incubated with horse radish peroxidase (HRP)-linked anti-rabbit or anti-mouse secondary antibodies (Boster, USA) at room temperature for 30 min followed by chromagen detection with DAB (Boster, USA). Hematoxylin (Boster, USA) was used as a counterstain. Colon cancer sections are known to exhibit positive RASSF1A expression and were used as positive controls. A subset of sections was incubated with no primary antibody (only PBS buffer) as a negative control.

Light microscopy was used to observe and image sections. A cell exhibiting cytoplasmic and/or cell membrane tan particles was considered positively labeled. Double blind and semi-quantitative methods were used to evaluate the results. Each specimen was separately scored for percentage of positive staining (0<5%, negative, 1 = 5-25%, 2 = 26-50%, 3 = 51-75% and 4>75%) and staining intensity (0 = none, 1 = light yellow, weak, 2 = yellow, moderate and 3 = tan, strong). Positive cell percentage scores multiplied by staining intensity scores were denoted as the dyeing integral [28].

All data were analyzed using the statistical software package SPSS11.5. Analysis of variance (ANOVA) was used to compare the dyeing integrals’ differences in different endometrial tissues and different ages as well as stages and grades in the carcinoma tissue. At-test was used to analyze the same indices between different depths of muscular infiltration in the carcinoma tissue. A p-value of <0.05 was taken as statistically significant. 

Methylation-specific PCR

HEC-1-B cells were collected from every group and used to assess the methylation levels of the RASSF1A promoter in HEC-1-B cells by methylation specific PCR (MSP). After extracting genomic DNA, bisulfite treatment was used following PCR amplification and gel electrophoresis. The objective product of the amplified PCR was 169 bp. The RASSF1A DNA sequence was obtained from the NCBI Human Genome Database. The non-methylated primer (forward: 5'-GGTTTTGTGAGAGTGTGTTTAG-3'; reverse: 5'-CACTAAACACAAACACACAAAC-3'), and methylated primers (forward: 5'-GGGTTTTGCGAGAGCGCG-3'; reverse: 5'-GCTAAACACACGCGAACC-3') were synthesized by Invitrogen (Life Technologies Corporation, USA). PCR thermocycling parameters were as follows: 95°C for 3 min, then 35 cycles, at 95°C for 30 s, 53°C for 30 s, 72°C for 45 s, followed by 72°C for 10 min and ending at a holding temperature of 4°C.

Fluorescence quantitative PCR

RASSF1A RNA sequences were obtained from the NCBI Human Genome Database. Primers were designed by Primer Premier5 and synthesized by Invitrogen (sense: 5'-GCCCTGCTGCGAAAGTTCT-3'; antisense: 5'-CCAACAGCTTCCGCAAGTACA-3') and the amplified PCR product was 100 bp. Total RNA from HEC-1-B cells was extracted using Trizol (TaKaRa, Japan) and cDNA was prepared using a commercially available reverse transcriptase kit (TaKaRa, Japan). Resulting cDNA was then analyzed by fluorescence quantitative PCR using SYBR MIX (QP-201) (TOYOBO, Japan) in an Eppendorf Mastercycler realplex. Results were the representative of three similar experiments. Data were calculated using the $2^{-\Delta\Delta C_t}$ formula. The statistical software package SPSS16.0 was used for data analysis and an ANOVA was used to compare RASSF1A RNA levels between each two of all the above groups. A p-value of <0.05 was taken as statistically significant.

Western blot

HEC-1-B cells were lysed using commercially available lysis buffer (QIAGEN, Germany) for 30 min and followed by six cycles of ultrasonication on ice. Total RASSF1A protein was quantified, separated using SDS-PAGE, and proteins were transferred onto PVDF membranes (Merck Millipore, Germany). Membranes were blocked with PBST using 5% (w/v) skim milk powder for 1 h at 37°C. Blots were then incubated with primary antibodies (1:400) at 4°C overnight, followed by incubation with the appropriate secondary antibody (1:4000) for 1 h at 37°C. Immunolabeled proteins were then detected using the gel quantitative image analysis system Quantity One 4.62 and regions of interest were analyzed to obtain integral optical densities (IOD). The housekeeping protein β-actin was used as an internal control for all experiments. The statistical software package SPSS16.0 was used for data analysis and an
ANOVA was used to compare RASSF1A protein levels between two of all the above groups. A p-value of <0.05 was taken as statistically significant.

**TUNEL staining to examine apoptosis rates of HEC-1-B cells**

HEC-1-B cells from each treatment group were inoculated on germfree slips for 72 h, after which they were fixed by formaldehyde for 25 min at 4°C. Cells were then washed by PBS, put into 0.2% TritonX-100 solution for 5 min, and washed again by PBS and equilibrium buffer. Slips were then incubated in 50 μL of TdT incubation buffer at 37°C for 60 min and subjected to 2 × saline-sodium citrate (SCC) buffer for 15 min. Five regions were randomly selected and 200 cells from these were imaged using fluorescence microscopy (magnification 200 ×). Imaged cells were then used to calculate the apoptosis index which was defined as: Apoptosis index (AI%) = apoptosis numbers/total × 100. The statistical software package SPSS16.0 was used for data analysis and an ANOVA was used to compare apoptosis rates between two of all the above groups. A p-value of <0.05 was taken as statistically significant.

**Xenograft of endometrial carcinoma in nude mice**

HEC-1-B cells were suspended in DMEM and injected subcutaneously (0.1 ml per mouse) in the right legs of five-week-old female BALB/c nude mice (Vital River Laboratory Animal Technology Co., Ltd) after adjusting for cell density (5 × 10⁷/ml). After nine days, 21 eligible nude mice (similar sizes of miliary nodules in the injected sites) were randomly divided into seven groups. These groups were exposed to one of the following drug combinations: Group 1 (5-Aza-CdR), Group 2 (cisplatin), Group 3 (medroxyprogesterone acetate), Group 4 (5-Aza-CdR with cisplatin), Group 5 (5-Aza-CdR with medroxyprogesterone acetate), Group 6 (cisplatin with medroxyprogesterone acetate), and Group 7 (control group, physiological saline only). The dosage of every drug was administered at 1 µg/g (0.2 ml) per mouse, once every three days by tail vein injection for a total of eight treatments. Both the longest and shortest diameter of the xenograft were measured with vernier calipers every three days by the same experimenter. Nude mice were sacrificed three days after drug withdrawal, after which tumors were dissected out and weighed. Volume was calculated according to the following formula: (V, mm³) = 1/2 (a × b²) and inhibitory rate was calculated according to the following formula (IR%) = [(Vcontrol-Vexperimental)/Vcontrol] × 100%. RASSF1A DNA methylation and RASSF1A protein expression were tested using MSP and Western blot, respectively. Xenograft cellular apoptosis was analyzed using TUNEL staining. The statistical software package SPSS19.0 was used for data analysis. Average values were expressed as mean ± the standard deviation (SD) and analyzed using a Student’s
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ANOVA and Fisher’s Exact Test were used to compare the means between groups across all categorical data. A p-value of <0.05 was taken as statistically significant. All experiments were performed at least three times.

**Results**

**Immunohistochemical analysis of RASSF1A protein expression**

As shown in Figure 1 (400 × magnification), RASSF1A protein was mainly expressed in the cytoplasm and/or cell membrane of different cells in the examined tissue. The staining intensity distributions for different endometrial tissues are shown in Figure 2. The contrast of staining integral in different endometrial tissues shown in Table 1. RASSF1A protein expression was significantly reduced in endometrial carcinoma tissues when compared to both atypical hyperplasia and normal endometrial tissue (both P< 0.0001). The difference in RASSF1A protein expression between atypical hyperplasia and normal tissue was not statistically significant (P = 0.692). There were also no significant differences in RASSF1A protein expression when compared to different clinical pathological factors such as age, grading, staging, myometrial invasion, or endometrial carcinoma pathological type (all P>0.05, Table 2).

**Table 1.** Staining integral contrast indifferent endometrial tissues

<table>
<thead>
<tr>
<th>Endometrial types</th>
<th>Cases (percentage)</th>
<th>Staining integral</th>
<th>Mean ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td>29</td>
<td>6-12</td>
<td>11.7143±1.40372</td>
</tr>
<tr>
<td>Atypical hyperplasia</td>
<td>21</td>
<td>6-12</td>
<td>11.5517±1.30931</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>51</td>
<td>0-6</td>
<td>2.1373±1.48350</td>
</tr>
<tr>
<td>Total</td>
<td>101</td>
<td>0-12</td>
<td>6.8317±4.97005</td>
</tr>
</tbody>
</table>

**Table 2.** Comparison of different ages, grading, staging, myometrial invasion, and pathological types of endometrial carcinoma

<table>
<thead>
<tr>
<th>Clinical pathology</th>
<th>N (%)</th>
<th>H-score (mean ± standard deviation)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>8 (15.68%)</td>
<td>2.7500±1.75255</td>
<td>0.418</td>
</tr>
<tr>
<td>41-50</td>
<td>18 (35.29%)</td>
<td>2.0556±1.39209</td>
<td></td>
</tr>
<tr>
<td>51-60</td>
<td>16 (31.37%)</td>
<td>2.2500±1.61245</td>
<td></td>
</tr>
<tr>
<td>≥61</td>
<td>9 (17.65%)</td>
<td>1.5556±1.13039</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td>0.873</td>
</tr>
<tr>
<td>G1</td>
<td>7 (13.7%)</td>
<td>2.0000±1.15470</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>31 (60.8%)</td>
<td>2.2258±1.70704</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>13 (25.5%)</td>
<td>2.0000±1.08012</td>
<td></td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td>0.433</td>
</tr>
<tr>
<td>I</td>
<td>38 (74.5%)</td>
<td>2.1579±1.51615</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>7 (13.7%)</td>
<td>2.5714±1.71825</td>
<td></td>
</tr>
<tr>
<td>III-IV</td>
<td>6 (10.8%)</td>
<td>1.5000±0.83666</td>
<td></td>
</tr>
<tr>
<td>Pathological type</td>
<td></td>
<td></td>
<td>0.520</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>37 (72.5%)</td>
<td>2.0541±1.54463</td>
<td></td>
</tr>
<tr>
<td>Adenosquamous Carcinoma</td>
<td>14 (27.5%)</td>
<td>2.3571±1.33631</td>
<td></td>
</tr>
<tr>
<td>Depth of myometrial infiltration</td>
<td></td>
<td></td>
<td>0.667</td>
</tr>
<tr>
<td>≤66</td>
<td>31 (60.8%)</td>
<td>2.0645±1.50412</td>
<td></td>
</tr>
<tr>
<td>&gt;1/2</td>
<td>20 (39.2%)</td>
<td>2.2500±1.48235</td>
<td></td>
</tr>
</tbody>
</table>

RASSF1 ADNA methylation status in HEC-1-B cells

RASSF1A promoter region in Group A was markedly hyper-
methylated and only produced a methylation product. Of these methylation products, those that were expressed decreased in a dose-dependent manner in response to 5-Aza-CdR treatment, as seen in Groups B, C, and D (Figure 3).

**Table 3. RASSF1A mRNA and protein expressions in HEC-1-B cells**

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein expression (48 h) (IOD value)</th>
<th>mRNA expression (24 h) (2−ΔΔCt value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.692±0.069 0.004378±0.00026</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.431±0.152 0.005102±0.00007</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.344±0.038 0.005461±0.00027</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>3.081±0.130 0.006942±0.00040</td>
<td></td>
</tr>
</tbody>
</table>

As shown in Table 3, there was no significant differences in RASSF1A mRNA levels between Groups A and B ($P_{ab} = 0.051$). However, there was a significant difference in mRNA levels between Groups A and C ($P_{ac} = 0.006$). When comparing experimental groups, there was a significant difference in RASSF1A levels between Groups C and D ($P_{cd} = 0.001$), although there was no significant difference between Groups B and C ($P_{bc} = 0.426$).

When RASSF1A protein expression was examined, all experimental groups showed a significant difference when compared with control Group A ($P<0.01$). As shown in Figure 4, RASSF1A protein expression increased in a dose-dependent manner in response to 5-Aza-CdR treatment in Groups B, C, and D.

**TUNEL analysis of HEC-1-B apoptosis**

As shown in Figure 5, TUNEL staining results showed a positive correlation between apoptosis (denoted by the amount of positive TUNEL staining) and 5-Aza-CdR concentration. We estimated apoptotic percentages as the following: 4% for Group A, 16% for B, 33% for C, and 46% for D. When compared with the control Group A, these differences were statistically significant across every experimental group ($P<0.01$). Additionally, all experimental groups were statistically different when examined using a pairwise comparison ($P<0.01$).

**In vivo RASF1A analysis in nude mice**

Tumor formation rate was approximately 95.83% in nude mice. As shown in Figure 6, the methylation band of the RASSF1A gene was prominent in Group 7 (saline control group), but less so in Groups 1, 4, and 5 (all treated with 5-Aza-CdR). In these groups, the prominent amplicon was shown to be the unmethylated band.

RASSF1A protein expression levels in xenograft tumors are shown in Figure 7. When compared with Group 7, Groups receiving 5-Aza-CdR showed significant differences (all $P<0.001$). There were no significant differences between the Control Group and those groups that were unexposed to 5-Aza-CdR (Group 2, $P = 0.085$, Group 3 ($P = 0.089$), and Group 6 ($P = 0.051$). There were no statistical differences found between the different 5-Aza-CdR treatment groups ($P>0.05$). Further detail on xenograft volumes, inhibitory rate, weight, cell apoptosis index, and protein expression are shown in Table 4. Before drug treatment, there were no significant differences between either volume ($P = 0.28$) or weight ($P = 0.95$) of transplantation tumors in all groups. After drug treatment and when compared with control Group 7, there were several significant changes: $P$ values of transplanted tumor weights and volumes respectively were 0.004 and 0.008 (Group 1),
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Figure 5. Representative images showing effects of different 5-Aza-CdR concentrations on HEC-1-B cellular apoptosis. A. 0 mol/L; B. 1.0 × 10⁻⁶ mol/L; C. 3.0 × 10⁻⁶ mol/L; D. 10 × 10⁻⁶/L. Original magnification 200 ×.

Figure 6. Representative images showing RASSF1A DNA methylation status of promoter regions of xenograft by different drugs. (U): unmethylated, (M): methylated; 1, AZA; 2, DDP; 3, MPA; 4, AZA+DDP; 5, AZA+MPA; 6, DDP+MPA; 7, normal saline.

0.001 and 0.001 (Group 3), and 0.0001 and 0.0001 for all the other groups. The difference in weight was statistically significant (P = 0.027) only between experimental Groups 1 and 5. There were no significant differences in volume between single drug treatment groups. In combined drug treatment groups, there were differences in volume between Groups 1 and 3, but not in Group 2.

As shown in the apoptosis index (AI) analysis presented in Table 4, there were significant differences in apoptosis in all experimental groups when compared with the control Group 7 (P = 0.014 in Group 2 and 0.0001 in all other groups). Additionally, there were statistically significant differences between single drug groups (P>0.05) or in drug combination groups (P>0.05). However, when these two types of treatment groups were compared with each other, there was a statistically significant difference (P<0.05).

Discussion

This study systematically sought to understand the role and mechanism of RASSF1A in the occurrence and development of endometrial carcinoma. To provide a more complete assessment, we chose both in vitro and in vivo approaches. Moreover, we also assessed the value of demethylation as a treatment strategy for endometrial carcinoma.

Our immunohistochemical results revealed that RASSF1A protein expression was not differentially expressed between normal and atypical hyperplastic tissues. However, it was reduced in endometrial carcinoma tissues, indicating that either low expression or inactivation of the RASSF1A gene might participate in the occurrence of endometrial carcinoma. This could then lead to malignant transformation from normal endometrium and atypical hyperplasia to endometrial carcinoma, similar to the role in thyroid carcinogenesis [29]. Although atypical hyperplasia is a precancerous lesion of endometrial carcinoma, there may be critical differences between their gene expression profiles. To this end, the gene expression profiles seen in atypical hyperplasia may be more similar to those normal tissues. However, further research will be needed to confirm this hypothesis. Although RASSF1A protein expression was not significantly correlated with various clinical factors of age or progression of endometrial carcinoma, RASSF1A protein expression in young patients appeared to be markedly weakened or missing altogether. This suggests that RASSF1A gene expres-
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Figure 7. Representative images showing RASSF1A protein expression in different xenograft groups. (U): unmethylated, (M): methylated; 1, AZA; 2, DDP; 3, MPA; 4, AZA+DDP; 5, AZA+MPA; 6, DDP+MPA; 7, normal saline.

Results obtained from the HEC-1-B and transplantation tumor experiments further confirmed that the RASSF1A promoter region had abnormal hypermethylation patterns in endometrial cancer samples. After demethylation using varying 5-Aza-CdR concentrations, we found that hypermethylation status could be gradually reversed and that the biological effects of this were proportional to the drug concentration. When compared with an untreated control group, the cell apoptosis index increased significantly in tumor cells. Critically, treatment with 5-Aza-CdR appeared to reduce this apoptotic index. Collectively, this demonstrates that hypermethylation of the RASSF1A promoter is a likely contributor to the cellular apoptosis seen in cancer. Experimentally, we found that the lowest effective drug concentration which could impact mRNA and protein expression was $3.0 \times 10^{-6}$ mol/L and $1.0 \times 10^{-6}$ mol/L and that drug action time may affect these two features differentially (24 h for mRNA and 48 h for protein). Therefore, we concluded that the effects of 5-Aza-CdR depended not only on its concentration, but also its exposure time. This can also be seen in previous work done with cervical cancer cells [30].

From the volume comparisons of transplanted tumors, it was clear that all experimental groups had markedly smaller tumors than controls. However, there were no detectable differences between monotherapies. Interestingly, we found that there were significant differences between monotherapy groups and drug combination groups, indicating that while 5-Aza-CdR, cisplatin, and medroxyprogesterone acetate could inhibit tumor growth, combinations of them could enhance their individual antitumor effects. To this end, a combination of 5-Aza-CdR and medroxyprogesterone acetate showed particularly strong therapeutic effects. Given these findings, we speculated that the progesterone receptor gene might also be susceptible to aberrant hypermethylation in endometrial adenocarcinoma, which could then recover relevant biological functions after demethylation. This theory is supported by some published reports, which have shown that expression of the progesterone receptor gene is decreased and abnormally hypermethylation in endometrial carcinoma [31-34].

Furthermore, cellular apoptosis resulting from transplantation tumors showed that all experimental groups had an increase in tumor cell death when compared to an untreated control group. Once again, although no obvious differences were observed between monotherapies, there were statistically significant differences between single drug groups and drug combination groups. In other words, drug efficacy
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Table 4. Volumes, inhibitory rate, weight, cell apoptosis index, and protein expression of xenograft groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Xenograft volume (mm$^3$) (mean ± standard deviation)</th>
<th>Inhibitory rate</th>
<th>Xenograft Weights (g)</th>
<th>Apoptosis index</th>
<th>Protein expression (IOD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before medication</td>
<td>After medication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>42.94±3.61</td>
<td>192.61±21.49</td>
<td>43.379%</td>
<td>1.39±0.16</td>
<td>2.27±0.13</td>
</tr>
<tr>
<td>2</td>
<td>43.84±3.26</td>
<td>97.63±38.89</td>
<td>71.481%</td>
<td>1.04±0.35</td>
<td>0.99±0.06</td>
</tr>
<tr>
<td>3</td>
<td>42.51±2.90</td>
<td>148.54±58.52</td>
<td>56.614%</td>
<td>1.26±0.08</td>
<td>0.98±0.15</td>
</tr>
<tr>
<td>4</td>
<td>42.57±1.68</td>
<td>64.19±15.70</td>
<td>81.249%</td>
<td>0.81±0.30</td>
<td>2.26±0.69</td>
</tr>
<tr>
<td>5</td>
<td>42.90±0.37</td>
<td>85.56±23.51</td>
<td>70.023%</td>
<td>0.71±0.32</td>
<td>2.17±0.38</td>
</tr>
<tr>
<td>6</td>
<td>42.57±1.68</td>
<td>88.84±25.03</td>
<td>74.051%</td>
<td>0.62±0.25</td>
<td>0.85±0.23</td>
</tr>
<tr>
<td>7</td>
<td>45.54±1.76</td>
<td>342.36±18.22</td>
<td>--</td>
<td>2.12±0.12</td>
<td>18.11%±0.69</td>
</tr>
</tbody>
</table>

appeared to have no effect on inducing transplantation tumor cell apoptosis, although combination drug treatment could enhance their individual effects. However, whether or not 5-Aza-CdR might be damaging nearby healthy cells remains to be determined.

Although we designed our experiments to keep conditions equal in every group, certain biases and errors were unavoidable, including less-than-optimal sample sizes. To this end, larger group sizes may be needed to confirm and evaluate our current conclusions. In addition, the occurrence and development of gynecologic cancers are complicated processes and involve a variety of oncogenes and antioncogenes [35, 36]. In effect, low \textit{RASSF1A} expression caused by hypermethylation may be just one of many factors leading to the development of endometrial carcinoma [37-39]. Additionally, the mechanisms of 5-Aza-CdR in inhibiting the growth of endometrial cancer and inducing tumor cells apoptosis may not only restore the function of the tumor suppressor gene \textit{RASSF1A}, but could also involve other mechanisms [38, 40]. These might include the progesterone receptor gene, as previously mentioned. Collectively, our research provides the basis for further work into the relationship between \textit{RASSF1A} and endometrial cancer, which we expect to yield new therapeutic targets for the treatment of endometrial carcinoma.

In conclusion, abnormal methylation in different promoter regions may be a key factor responsible for the decreased expression or dysfunction of important tumor suppressor genes. Additionally, de-methylation agents such as 5-Aza-CdR offer hope as new candidates for the treatment of endometrial cancer and should be explored as a new method of chemosensitization in the treatment of human endometrial carcinomas.

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**Disclosure of conflict of interest**

None.

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**References**


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[28] Oreshkov S, Babic D, Kalafatic D, Barisic D, Beketic-Oreskovic L. A significance of immunohistochemical determination of steroid receptors, cell proliferation factor Ki-67 and protein
Investigating RASSF1A hypermethylation and endometrial carcinoma


