

RESEARCH ARTICLE

Aberrant Methylation of RASSF2A in Tumors and Plasma of Patients with Epithelial Ovarian Cancer

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Abstract

Objective: The tumor suppressor gene, Ras-association domain family (RASSF)2A, is inactivated by promoter hypermethylation in many cancers. The current study was performed to evaluate the methylation status of RASSF2A in epithelial ovarian cancer (EOC) tissues and plasma, and correlations with gene expression and clinicopathologic characteristics. **Method:** We detected methylation of the RASSF2A gene in tissues and corresponding plasma samples from 47 EOC patients and 14 patients with benign ovarian tumors and 10 with normal ovarian tissues. The methylation status was determined by methylation-specific PCR while gene expression of mRNA was examined by RT-PCR. The EOC cell line, SKOV3, was treated with 5-aza-2'-deoxycytidine (5-aza-dC). **Results:** RASSF2A mRNA expression was significantly low in EOC tissues. The frequency of aberrant methylation of RASSF2A was 51.1% in EOC tissues and 36.2% in corresponding plasma samples, whereas such hypermethylation was not detected in the benign ovarian tumors and normal ovarian samples. The expression of RASSF2A mRNA was significantly down-regulated or lost in the methylated group compared to the unmethylated group ($p < 0.05$). After treatment with 5-aza-dC, RASSF2A mRNA expression was significantly restored in the Skov3 cell line. **Conclusion:** Epigenetic inactivation of RASSF2A through aberrant promoter methylation may play an important role in the pathogenesis of EOC. Methylation of the RASSF2A gene in plasma may be a valuable molecular marker for the early detection of EOC.

Keywords: Epithelial ovarian cancer - RASSF2A - hypermethylation - plasma

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Introduction

Epithelial ovarian cancer (EOC) is the third leading type of female gynecologic carcinomas, and has a high mortality rate (Siegel et al., 2012). Because of the non-specific symptoms and the lack of early diagnostic methods, EOC is extremely difficult to diagnose at an early stage. Nearly 70% of patients are diagnosed at an advanced stage (FIGOII-IV), with a survival rate of 30-40% (Cannistra et al., 1993; Barnholtz-Sloan et al., 2003; Qiu H et al., 2011); however, if diagnosed at a localized stage (FIGOI), the 5-year survival rate is 93% (Schlaerth et al., 2009). Therefore, a study on the molecular mechanism underlying ovarian cancer progression, including a search for a related target gene, is important for early diagnosis and effective therapy for ovarian cancer.

It is now increasingly recognized that both genetic and epigenetic events play a role in the development of ovarian cancer, with epigenetic changes occurring early in the carcinogenic process (Wei et al., 2006; Jones et al., 2007). There is evidence indicating that epigenetic silencing of gene expression by promoter hypermethylation is a major mechanism for the inactivation of tumor suppressor genes (TSGs) in cancer (Mutskov et al., 2002; Herman et al., 2003; Qiu H et al., 2011). Promoter hypermethylation is

a common epigenetic mechanism for TSG inactivation in human cancer and a promising target for molecular detection (Jones et al., 1999).

RAS proteins are key signal transducers for various important pathways, such as PI-3K, MAPK, and Rho GTPases, which interact with a wide range of effector proteins to regulate various biological processes, such as proliferation, differentiation, apoptosis, cell cycle arrest, and senescence (Spandidos et al., 2002). RASSF is defined as a negative effector of RAS and currently includes 10 members (RASSF1-RASSF10). The RASSF members all contain a Ras-association domain and are thought to function as tumor suppressors by regulating cell cycle, apoptosis, and microtubule stabilization (Richter et al., 2009).

Epigenetic inactivation of RASSF1 has been well-characterized in a wide variety of tumors. Methylation of the RASSF1A promoter is described as an early and frequent event in tumorigenesis, thus RASSF1A can serve as a useful diagnostic marker in cancer screening (Dammann et al., 2000; Dammann et al., 2001; Liu WJ et al., 2013), including ovarian cancer (Agathangelous et al., 2001; Yoon et al., 2001; Rathi et al., 2002; Ibanez et al., 2004; Makarla et al., 2005; Choi et al., 2006; Ma et al., 2009; BonDurant et al., 2011; Bhagat et al., 2012).

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Recently, RASSF2 was identified as a new negative effector of RAS protein. The RASSF2 gene contains 11 exons that span ~43kb at chromosome 20p13, and has 3 different isoforms (RASSF2A, RASSF2B, and RASSF2C). All of these isoforms contain Ras association (RA) domains, but only RASSF2A has an associated CpG island (Hesson et al., 2005). Down-regulation of RASSF2A by promoter hypermethylation has been shown in different tumor cell lines and primary tumors, which suggests that RASSF2A participates in carcinogenesis (Vos et al., 2003; Akino et al., 2005; Endoh et al., 2005; Park et al., 2007; Zhang et al., 2007; Ren et al., 2009).

Limited data regarding the methylation status in EOC are available. In the present study we determined the level of RASSF2A expression in EOC and the methylation status of RASSF2A in EOC tissues and plasma, and analysed the correlation between RASSF2A and clinicopathologic characteristics.

Materials and Methods

Patients and samples

Forty-seven EOC patients were enrolled. Fourteen patients with benign ovarian tumors and 10 patients with normal ovarian tissues were included. All of the patients underwent surgical treatment in the Department of Gynecology and Obstetrics at Liaocheng People's Hospital (Shandong, China) between February 2012 and October 2013. The diagnosis was confirmed histopathologically in all cases, and all samples were obtained prior to chemotherapy or radiotherapy. Exclusion criteria were pregnancy, chronic or acute viral infection, an immunocompromised state, and other cancers. All patients provided consent, and study approval was obtained from the Ethics Committee.

Histologic classifications were established according to the World Health Organisation (WHO) criteria, and tumour stages were established according to the International Federation of Gynecology and Obstetrics (FIGO). Stages I-II was regarded as an early tumor stage, whereas stages III-IV was identified as an advanced tumor stage.

All of the cases yielded sufficient fresh tissue and paired plasma samples for DNA and RNA extraction. The plasma samples were collected before surgery. The surgically-excised tissues were immediately frozen in liquid nitrogen and stored at -80 °C.

Cell line

The EOC cell line, SKOV3, was used in this study. The cell line was cultured in appropriate medium containing 10% fetal bovine serum at 37 °C in 5% CO₂.

RT-PCR

Reverse transcription-PCR analysis was used to determine RASSF2A expression. Total RNA in preserved frozen tissues and the cell line was isolated with Trizol reagent according to the manufacturer's instructions. A cDNA was synthesized using MMLV reverse transcription, then assayed by PCR, which consisted of an initial denaturation for 10 min at 95 °C, followed by 35

cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The RASSF2A-specific primers were as follows: forward primer, 5'-GCGCCTAGAACGTGTTTTTC-3'; and reverse primer, 5'-ACTAGGCGTCCTCACATTGC-3'. The primers used for the housekeeping gene, GAPDH, were as follows: 5'-CAACGGATTTGGTCGTATT-3'; and 5'-CACAGTCTTCTGGGTGGC-3'. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). The amplified products were separated on 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

DNA extraction and bisulfite modification

Genomic DNA from frozen preserved tissues and corresponding plasma samples was extracted by proteinase K digestion and a phenol/chloroform extraction method (Darehdori et al., 2012). One microgram of the genomic DNA was denatured by NaOH and modified by sodium bisulfite and hydroquinone. Bisulfite-treated DNA was purified using a Wizard DNA clean-up system (Promega, Madison, WI, USA) following the manufacturer's instructions. The DNA was treated with NaOH, precipitated by ethanol, and eluted into 50 µl of distilled water. The final products were stored at -80 °C for later use.

Methylation-specific PCR (MSP)

After bisulfite treatment, all unmethylated cytosines were converted to uracils, but methylated DNA was protected. The methylation status in the promoter region of RASSF2A was determined by MSP. Modified genomic DNA was amplified by using two different sets of primers specific for unmethylated and methylated RASSF2A promoters. The primers for the methylated reaction were as follows: sense, 5'-GTTTCGTCGTCGTTTTTTAGGCG-3'; and antisense 5'-AAAAACCAACGACCCCCGCG-3'. The primers for unmethylated were as follows: sense, 5'-AGTTTGTTGTTGTTTTTTAGGTGG-3'; and antisense, 5'-AAAAAACAACAACCCCCACA-3' (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.).

PCR reactions were performed using Hotstart Taq DNA polymerase (Qiagen, Valencia, CA, USA). PCR amplification was as follows: initial denaturation for 10 min at 95 °C; 35 cycles at 95 °C for 30 s, 58 °C for 30 s (MSP) or 54 °C for 30 sec (USP), and 72 °C for 30 s; and a final extension at 72 °C for 10 min. Normal lymphocyte DNA methylated in vitro with SssI methylase (New England Biolabs, Beverly, MA, USA) was used as a positive control (M+). Normal lymphocyte DNA as a negative control (U+) and water replacing DNA was used as a blank control. Products were subjected to electrophoresis on a 2% agarose gel.

Cell line and 5-aza-dC treatment

The SKOV3 cell line was treated with 5-aza-dC (Sigma, St. Louis, MO, USA). The cells were continuously exposed to 10 µM 5-aza-dC for 72 h. Mock-treated cells were cultured with an equivalent volume of PBS

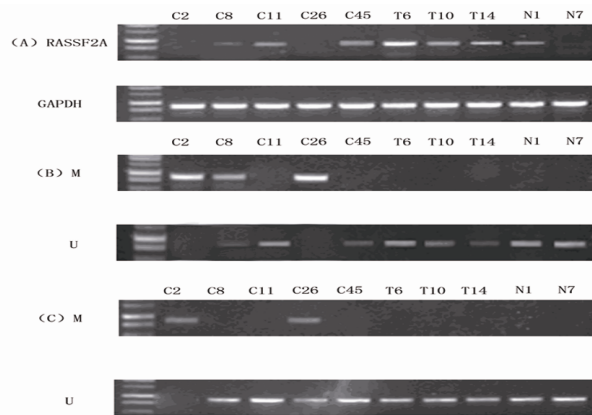


Figure 1. RASSF2A mRNA Expression and Promoter Hypermethylation Status. (A) The expression of RASSF2A in 47 primary EOC tissues, 14 benign ovarian tumors, and 10 normal ovarian samples analyzed by RT-PCR. RASSF2A mRNA expression was significantly lower in EOCs. C, EOC; T, benign ovarian tumors; N, normal ovarian samples. (B) Promoter hypermethylation status of the RASSF2A gene in ovarian tissues was examined by MSP. M, MSP; U, unmethylated-specific PCR. (C) Promoter hypermethylation status of the RASSF2A gene in plasma samples was also examined by MSP

alone. The medium and drugs were replaced every 24 h. Seventy-two hours after seeding, the cells were harvested. Total RNA was isolated, and RT-PCR was performed to determine the expression of RASSF2A, as described above.

Statistical Analysis

All statistical tests were performed using SPSS software (version 13.0, SPSS, Inc., Chicago, IL, USA). A Fisher's exact test and/or test were used to compare RASSF2A mRNA expression and methylation status among the cases, and with respect to various clinicopathologic characteristics. Pearson correlation analysis was used to evaluate the relationship between RASSF2A mRNA expression and methylation status in tissues and plasma samples. The differences in RASSF2A mRNA expression in RASSF2A-methylated tumors versus unmethylated tumors and before versus after 5-aza-dC treatment were analyzed by t-tests. A p value < 0.05 was regarded as statistically significant.

Results

RASSF2A mRNA expression in EOC tissues

RASSF2A expression was examined in 47 primary EOC tissues, 14 benign ovarian tumors, and 10 normal ovarian samples by RT-PCR. RASSF2A mRNA expression was absent in 42.6% (20/47) of EOCs. In contrast, mRNA was expressed in 85.7% (12/14) of the benign ovarian tumors and 90% (9/10) of the normal ovarian samples. RASSF2A mRNA expression was significantly lower in EOCs compared with benign ovarian tumors or normal ovarian samples ($p < 0.05$; Figure 1A).

Promoter hypermethylation status of the RASSF2A gene in EOC tissues

To determine whether or not the inactivation of

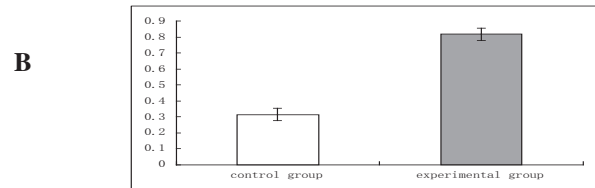
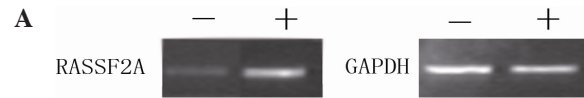


Figure 2. RASSF2A mRNA Expression after Treatment with 5-aza-dC. (A) RASSF2A mRNA expression was significantly restored in the SKOV3 cell line after treatment with 5-aza-dC for 3 days. (B) RASSF2A expression was calculated relative to that of GAPDH and expressed as the percentage normalized to the expression of the normal ovarian tissue

Table 1. Correlation Between the Expression and Methylation of RASSF2A in EOC Tissues

RASSF2A expression	RASSF2A promoter methylation		χ^2	r	p
	M	U			
+	8	19	11.665	-0.498	0.001
-	16	4			

RASSF2A is related to promoter hypermethylation in EOC tissues, we determined the methylation status of the RASSF2A gene by MSP. We found that the promoter was hypermethylated in 51.1% (24/47) of the RASSF2A genes in EOCs. In contrast, no RASSF2A promoter hypermethylation existed in benign ovarian tumors and normal ovarian samples. Thus, the RASSF2A promoter hypermethylation occurred exclusively in EOCs (EOCs vs. benign ovarian tumors and normal ovarian samples, $p < 0.05$; Figure 1B).

Combined with previous RT-PCR results, there was generally a negative correlation between hypermethylated RASSF2A and mRNA expression. RASSF2A mRNA expression was detected in most of the unmethylated EOC tissues (19/23). In contrast, RASSF2A expression was not detected in the RASSF2A-methylated EOCs (16/24), with the exception of 8 tissues. The expression of RASSF2A mRNA was significantly more down-regulated in the hypermethylated groups (0.0558 ± 0.0456) than the non-methylated groups (0.1560 ± 0.0431 , $p < 0.05$; Table 1).

To further demonstrate that hypermethylation is directly responsible for the inactivation of RASSF2A, the SKOV3 cell line was treated with $10 \mu\text{M}$ of 5-aza-dC for 3 days, then RASSF2A mRNA expression was determined by RT-PCR. After treatment, RASSF2A mRNA expression was significantly restored in the SKOV3 cell line compared with the non-treated cells (Figure 2).

Clinicopathologic significance of RASSF2A promoter hypermethylation in EOC tissues

To determine whether or not the promoter hypermethylated status of the RASSF2A gene is associated with the clinicopathologic characteristics of the

Table 2. Correlation Between the Promoter Hypermethylated Status of the RASSF2A Gene and Clinicopathologic Characteristics in EOC Tissues

Clinicopathologic parameters	n	RASSF2A promoter methylation		χ^2	p
		M	U		
Age					
<50 y	18	6	12	3.67	0.055
≥50 y	29	18	11		
Type of tumor				0.2	0.905
Serous	23	12	11		
Mucinous	15	7	8		
Endometrioid	9	5	4		
FIGO stage				0.521	0.471
Early	22	10	12		
Advanced	25	14	11		
Histopathologic grade				0.563	0.755
Grade 1	5	2	3		
Grade 2	9	4	5		
Grade 3	33	18	15		
Lymph node metastasis				0.521	0.471
Present	22	10	12		
Absent	25	14	11		

EOC patients, we compared the promoter hypermethylated status of the RASSF2A gene with patient age, FIGO stage, type of tumor, histopathologic grade, and lymph node metastasis. RASSF2A methylation was more frequent in the group ≥ 50 years of age (62.07%) than the group < 50 years of age (33.33%); there were no significant differences. In addition, no significant differences were observed between methylated and unmethylated patients with respect to other clinicopathologic characteristics (Table 2).

Promoter hypermethylation status of the RASSF2A gene in EOC plasma samples

To confirm whether or not analysis of the circulating cell-free DNA in serum might be a promising non-invasive diagnostic tool for the early detection of cancer, and to determine whether or the methylation profiles of serum matched EOC tissues, we also determined the methylation status of the RASSF2A gene by MSP in corresponding plasma samples. The frequency of serum RASSF2A hypermethylation was 36.2% (17/47) in EOC patients. At the same time, no serum RASSF2A promoter hypermethylation existed in benign patients and healthy controls. Moreover, the serum methylation profiles were included in the tissue methylation profiles in EOCs, and there was a positive correlation (Figure 1C).

Clinicopathologic significance of RASSF2A promoter hypermethylation in EOC plasma

We also evaluated the correlation between the promoter hypermethylated status of the RASSF2A gene in EOC plasma and clinicopathologic characteristics; there was no significant correlation (Table 3).

Discussion

In the current study, either in tissues or the corresponding

Table 3. Correlation Between the Promoter Hypermethylated Status of the RASSF2A Gene and Clinicopathologic Characteristics in EOC Plasma

Clinicopathologic parameters	n	RASSF2A promoter methylation		χ^2	p
		M	U		
Age					
<50 y	18	4	14	2.458	0.117
≥50 y	29	13	16		
Type of tumor				0.307	0.858
Serous	23	9	14		
Mucinous	15	5	10		
Endometrioid	9	4	5		
FIGO stage				3.237	0.072
Early	22	5	17		
Advanced	25	12	13		
Histopathologic grade				1.007	0.604
Grade 1	5	2	3		
Grade 2	9	3	6		
Grade 3	33	12	11		
Lymph node metastasis				0.339	0.56
Present	22	7	15		
Absent	25	10	15		

plasma samples, RASSF2A hypermethylation was detected in EOCs, while such hypermethylation was absent in the benign ovarian tumors and normal ovarian samples. The frequency of aberrant methylation ranged from 51.1% in tissues to 36.2% in corresponding plasma samples. We also examined the expression of RASSF2A mRNA and showed that EOC with promoter hypermethylation had reduced mRNA expression. In contrast, significantly increased RASSF2A mRNA expression was demonstrated in the EOC cell line after treatment with 5-aza-DC. There have been no data published on hypermethylation of the RASSF2A promoter in EOCs. We conclude that promoter hypermethylation of RASSF2A may be involved in transcription inactivation of RASSF2A expression in EOC. Epigenetic inactivation of RASSF2A through aberrant promoter methylation may play an important role in the formation of EOC, and assessment of RASSF2A methylation status may be a potentially useful biomarker to enhance the diagnosis of EOCs.

As a tumor suppressor gene, RASSF2A regulates the Ras signaling pathway. RASSF2A promotes apoptosis and cell cycle arrest, as well as inhibition of growth (Zhang et al., 2006; Maruyama et al., 2008), which binds directly to K-RAS in a guanosine 5'-triphosphate-dependent manner via the RAS effector domain. RASSF2A was cloned from a brain cDNA library and directly sequenced, confirming the genomic gene structure. RASSF2A shared 28% homology with the known Ras-effector/tumor suppressor RASSF1A (Hesson et al., 2005). Epigenetic inactivation appears to be a major mechanism for the loss of RASS2A expression (Zhang et al., 2006).

Promoter hypermethylation of RASSF2A was detected in 51.1% (24/47) of EOC tissues in the current study. Thus, promoter hypermethylation of RASSF2A is a frequent genomic event in EOCs. It has been reported that hypermethylation of RASSF2A is generally rare in normal, non-neoplastic tissues (Hesson et al., 2005). We analyzed

14 benign ovarian tumors and 10 normal ovarian tissues in parallel with EOC tissues. No RASSF2A promoter hypermethylation was detected in benign ovarian tumors or normal ovarian tissues. This result clearly indicated that RASSF2A promoter hypermethylation in EOCs is tumor-specific.

Previous studies have shown that aberrant hypermethylation occurring in promoter CpG islands is a significant mechanism of TSG silencing, and in certain cases, may be the only mechanism (Costello et al., 2001; Herman et al., 2003). Therefore, we investigated hypermethylation of the RASSF2A gene. RT-PCR was also performed to detect mRNA expression of RASSF2A in EOCs. There was a significant concordance between RASSF2A promoter methylation status and expression in EOCs. Of note, tumors with RASSF2A methylation showed weak RASSF2A expression in comparison to tumors with RASSF2A unmethylation. Treatment with 5-aza-dC leads to re-expression of RASSF2A in an EOC cell line. In this context, the aberrant methylation of RASSF2A may be one of the mechanisms by which gene transcription is inactivated in EOCs. In addition, methylation-mediated inactivation is potentially a reversible phenomenon (Dong et al., 2013). Turning this process around and up-regulating RASSF2A may prevent or reverse the malignant and metastatic phenotype, therefore serving as a novel therapeutic option for EOCs.

In endometrial carcinomas, RASSF2A hypermethylation was found more frequently in samples from older patients (>45 years of age, $p=0.041$; Liao et al., 2008). A similar observation has been reported in colorectal cancer (Park et al., 2007). Age-related methylation changes are involved in the development of cancer in elderly people. In EOCs there is an increasing trend in the frequency of RASSF2A methylation with increasing age, but no significant association was found.

In nasopharyngeal carcinoma and gastric cancer, a high frequency of RASSF2A methylation has been observed and correlated with lymph node metastasis (Zhang et al., 2006; Maruyama et al., 2008). RASSF2A was involved in tumor invasion and metastasis led by RAS (Zhang et al., 2006). In the current investigation, there was no significant correlation of RASSF2A methylation with lymph node metastasis and FIGO stage. The tumor-specific hypermethylation of RASSF2A can be detected in early- and advanced-stage EOCs, suggesting that RASSF2A gene promoter methylation plays an important role in the early development of EOC. Hence, RASSF2A methylation can serve as a potential biomarker for the early diagnosis of EOC.

Patients with early- and advanced-stage cancer have abnormally high levels of circulating DNA in the serum or plasma compared to healthy patients or those with non-malignant diseases (Perlin et al., 1972; Leon et al., 1977). Based on plasma sample findings, we have shown that RASSF2A promoter hypermethylation occurs in EOCs. Moreover, the serum methylation rate was less than the methylation rate in tissues. This is likely due to a loss in extraction and bisulfite conversion, instability, and a high background of normal DNA (Zhang et al., 2013); however, the serum DNA methylation profiles were fully

included in the tissue methylation profiles in EOCs. There was also good agreement between the serum and tissue methylation rates. This study indicated that detection of promoter methylation in plasma specimens could potentially provide a simple, non-invasive, and sufficiently sensitive method for EOC screening.

This study was limited by the small number of tissue and plasma samples from patients with EOCs. In the future, we will continue to verify the value of RASSF2A methylation in detecting EOCs and investigate the potential application in monitoring the prognosis during follow-up and predicting responses to chemotherapy and radiotherapy.

In conclusion, our study showed that the high frequency of promoter hypermethylation of the RASSF2A gene contributes to the loss of gene expression in primary EOCs. Furthermore, the results suggest that inactivation of the RASSF2A gene due to hypermethylation in the promoter region may play a critical role in tumorigenesis of ovarian carcinomas. Monitoring RASSF2A promoter methylation in plasma may also provide useful information for the early detection of EOCs.

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