Research Article

Down Regulation of RASSF1a, APC and GSTP1 is Associated with Epithelial Ovarian Carcinoma Tumorigenesis

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Abstract

Ovarian cancer is the third leading gynecological malignancy in India with a high mortality rate. The high mortality of the disease is attributed to the late stage diagnosis of the disease. Silencing or down regulation of Tumor Suppressor genes is known to be an early event during tumorigenesis and is known to regulate several cellular events including DNA damage, proliferation and apoptosis. In this study, we have evaluated the mRNA expression of three TSG-RASSF1a, APC and GSTP1 in ovarian carcinoma tissues. We report here that 72/76 samples (94.73%) samples show a down regulation of RASSF1a gene, 47/76 (61.84%) sample show down regulation of APC and 51/76 (67.10%) samples showed down regulation of GSTP1. 100% of the benign samples showed downregulation of RASSF1a and GSTP1 and 77.77% of benign samples showed downregulation of APC gene, which indicates that the down regulation of these TSGs happen early in tumorigenesis and contribute to disease progression.

Development of molecular markers that can detect such early changes that occur during tumorigenesis can be utilized as a potential maker for diagnosis and prognosis of ovarian carcinoma. This can also shed light in identifying and designing suitable treatment strategies that can be more effective in management of the disease.

Keywords: RASSF1a; APC; GSTP1; Epithelial Ovarian Cancer; Diagnosis; Gene expression

Introduction

Ovarian Carcinoma (OC) arising from the ovarian surface epithelium accounts to about 95% of all ovarian malignancies. It is predicted that annually worldwide, 230 000 women will be diagnosed and 150 000 will die of the disease implicating a high death to incidence ratio. It stands as the seventh most commonly diagnosed cancer among women in the world and the third leading gynecological malignancy in India, with 46% survival rate 5 years after the diagnosis [1-3]. The main factor for increased mortality for EOC is the late stage detection of the disease. The survival rate in patients with early stage presentation is 92% in contrast to 29% survival rate in patients with late stage presentation [3,4]. Unfortunately over 75% of the patients are diagnosed at late stages where the disease would have already metastasized within the peritoneal cavity. Diagnosis of ovarian cancer includes the estimation of serum CA125 and pelvic ultrasonography. There are no molecular methods available for the precise detection of the disease in the early stages [1].

Inactivation or silencing of tumor suppressor genes (TSG) is known to be an early event in the initiation and progression of many cancers including EOC. TSGs are known to be involved in coding growth constraining proteins whose loss of function leads to deregulated cellular proliferation leading to tumorigenesis [5,6]. It has been reported that there are about 1217 human TSGs that are identified whose silencing is known to contribute to tumorigenesis[7]. Along with their growth constraining function, Tumor suppressor genes are known to be involved in several cellular processes such as DNA damage repair, induction of apoptosis and suppression of metastasis.

RASSF1a is a TSG located on chromosome 3p21.3, encodes for the Ras-super family of GTP-ases that are essential for intracellular signal transduction. RASSF1a modulates several cellular pathways including apoptotic pathway and tubulin dynamics. RASSF1a is also known to cause the repression of cyclin A and cyclin D1 to mediate cell cycle arrest [3,8,9]. RASSF1a is known to be silenced via epigenetic mechanism rather than mutational events. The silencing of this gene is known to be attributed in several cancer including lung, breast, prostate, renal and ovarian cancers [10-16].

APC1 located on chromosome 5q21-q22 is found to be expressed in many fetal and adult epithelial cells [17]. APC1 is known to play important roles in cell migration, adhesion, cytoskeleton organization and regulation of cellular proliferation [18,19]. Reports also suggest that APC1 is also involved in apoptosis and cell cycle arrest from Go/G1 to S phase [20,21]. Genetic alterations such as mutations are known to cause silencing of APC1 in many cancer

types [22,23]. However, in ovarian cancer APC1 gene silencing is attributed to epigenetic silencing through promoter hypermethylation [18,24-26].

The *GSTP1* gene is localized on chromosome 11q13 is a cytosolic detoxifying enzyme playing a critical role in maintaining cell integrity and protecting DNA from genotoxic stress. GSTP1 is also attributed to regulate the apoptosis signalling pathway. It has been reported that loss of GSTP1 expression, enhances cells to acquire additional genetic alterations that contribute to cancer progression.

There stands an indispensible need to diagnose ovarian cancer at the initial stages which could there by contribute to better management of the disease in addition to identifying new therapeutic targets for treatments. In the present study, we have assessed the mRNA expression of RASSF1a, APC1 and GSTP1 in primary ovarian cancer, low malignant potential and benign tissues in an attempt to assess if these could act as potential markers for diagnosis and prognosis.

Methods

Patient Samples

76 Ovarian tumor tissue was obtained (51 malignant, 16 Low Malignant Potential-LMP and 9 benign cyst adenoma) from patients reporting to Kidwai Memorial Institute of Oncology, Bangalore, India. All the samples were verified histologically by a pathologist before including in the study. Histological classification was established as per the WHO criteria and staged according to the FIGO classification. Normal ovarian tissue was obtained from patients undergoing salphingo-oopherctomy. The study was approved by the institutional scientific review board and medical ethics committee and informed consent was obtained from all patients prior to sample collection.

RNA isolation and cDNA preparation

Tumor samples were collected in RNAlater™ after histopathological confirmation. Total RNA was extracted from 30 mg of tissue using RNeasy mini kit (Qiagen, CA, USA) following the manufacturer's protocol. The RNA was quantified using Eppendorf biospectrophotometer kinetics™ and 1µg of RNA was used for cDNA preparation. cDNA was prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The conditions for cDNA preparation used are mentioned in table 1.

	Step1	Step2	Step3	Step4
Temperature	25^0C	37^0C	85^0C	4^0C
Time	10mins	120mins	5mins	∞

Table 1: cDNA conversion

Gene Expression Analysis

Gene expression of RASSF1a, APC1 and GSTP1 was quantified by TaqMan chemistry on StepOnePlus™ Real-Time PCR system (Applied Biosystem, CA, USA). Gene-specific primers and probe of RASSF1a (Hs00200394_m1), APC1 (Hs01568282_m1) and GSTP1 (Hs00168310_m1) were available as TaqMan gene expression assays (Applied Biosystems). The 18S rRNA (Hs99999901_s1) was amplified and was used as an endogenous control in the quantification. The gene expression was expressed as fold change. The gene expression values were correlated with various clinicopathological parameters.

Statistical Analysis

Chi square test and Fischer exact test (two- tailed) was used for analysis and a p-value <0.05 was considered to be statistically significant.

Results

Gene Expression Analysis of RASSF1a, APC and GSTP in Ovarian Cancer

Gene Expression of *RASSF1a*

Gene expression analysis of revealed that 72 cases of the total 75 samples analyzed had down regulation of RASSF1a. RASSF1a was downregulated in 48/51 (94.10%) malignant samples, 15/16 (93.75%) LMP and 9/9 (100%) benign samples analyzed. The samples were analyzed for statistical significance using Fischer exact test and the data correlated significantly among the group compared. The above data is summarized in table2. The gene expression plot for RASSF1a of tumor samples in comparison to normal samples is depicted below.

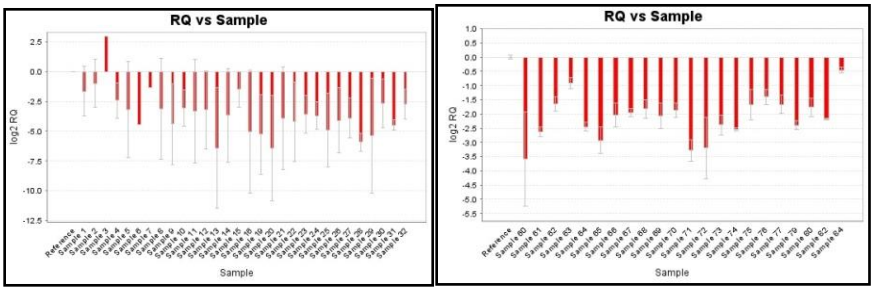

Figure 1: Representative Gene expression fold change of RASSF1a.

Expression of RASSF1a	**Tumor type**			
	Malignant (51)	**LMP (16)**	**Benign(09)**	**Normal (5)**
≥ 1 RQ	03/51 (5.88%)	1/16(6.25%)	0/9(0%)	5/5(100%)
<1 RQ	48/51 (94.11%)	15/16 (93.75%)	9/9(100%)	0/5
p-Value	**<0.0001***	**0.0003***	**0.0005***	

Table 2: Representation of RASSf1a gene expression in malignant, LMP, benign and normal tissue.

Gene expression of APC

APC expression was downregulated in 47/76 samples analyzed. 31/51(60.70%) malignant, 9/16 (56.25%) LMP and 7/9 (77.77%) benign samples showed downregulation of the APC1 mRNA. The p-value was found to be 0.014 for Malignant, 0.045 for LMP, 0.021 for benign samples and is represented in table3.

The gene expression plot of APC1 gene in tumor in comparison with normal samples is shown below.

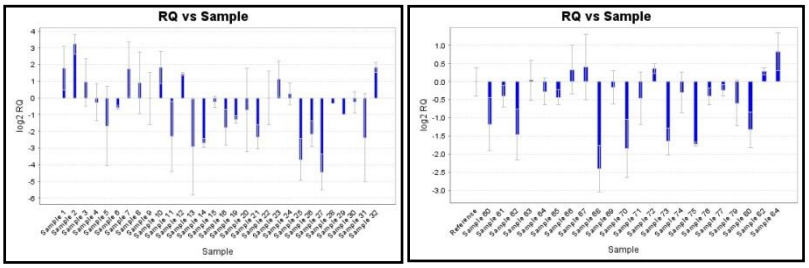

Figure 2: Representative gene expression fold change of APC

Expression of APC	**Tumor type**			
	Malignant (51)	**LMP (16)**	**Benign(09)**	**Normal (5)**
≥ 1 RQ	20/51 (39.21%)	07/16(43.7%)	02/9(22.22%)	5/5(100%)
<1 RQ	31/51(60.70%)	9/16 (56.2%)	7/9(77.77%)	0/5
p-Value	**0.014***	**0.045***	**0.021***	

Table 3: Representation of APC gene expression in malignant, LMP, benign and normal tissue

Gene Expression of *GSTP1*

51/76 samples showed down regulation of the GSTP1 gene, of which 29/51 (56.80%) malignant, 13/16 (81.25%) LMP and 9/9 (100%) benign samples showed down regulation. The gene expression plot of GSTP1 in tumor and normal samples is depicted in the plot below. The data with p-value is represented in Table 4.

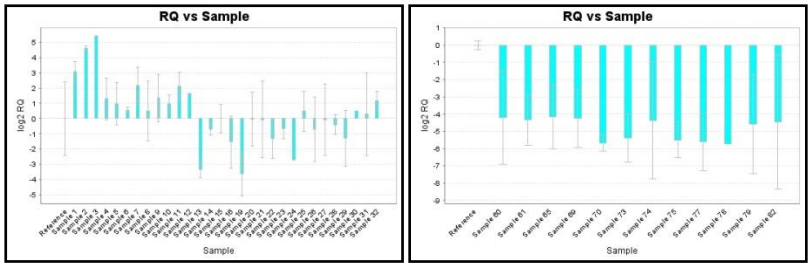

Figure 3: Representative gene expression plot of GSTP1.

Expression of GSTP	**Tumor type**			
	Malignant (51)	**LMP (16)**	**Benign(09)**	**Normal (5)**
≥ 1 RQ	22/51 (43.13%)	03/16(18.75%)	0/9(0%)	5/5(100%)
<1 RQ	29/51(56.80%)	13/16 (81.25%)	9/9 (100%)	0/5
p-Value	**0.021***	**0.002***	**<0.001***	

Table 4: Representation of GSTP1 gene expression in malignant, LMP, Benign and normal tissue.

37 of the 76 (48.68%) samples analyzed had low expression for all the three genes. 23 samples showed low expression of at least two genes (Table 5). This suggests that the silencing of TSG is an important contributor for tumor formation and progression.

SAMPLE	**RASSF1A**	**APC**	**GSTP**
1	LOW	HIGH	LOW
2	LOW	HIGH	LOW
3	HIGH	HIGH	HIGH
4	LOW	LOW	HIGH
5	LOW	LOW	HIGH
6	LOW	LOW	HIGH
7	LOW	HIGH	HIGH
8	LOW	HIGH	HIGH
9	LOW	LOW	HIGH
10	LOW	HIGH	HIGH
11	LOW	LOW	HIGH
12	LOW	HIGH	HIGH
13	LOW	LOW	LOW
14	LOW	LOW	LOW
15	LOW	LOW	LOW
16	LOW	LOW	LOW
17	LOW	LOW	LOW
18	LOW	LOW	LOW
19	LOW	LOW	LOW
20	LOW	HIGH	LOW
21	LOW	HIGH	LOW
22	LOW	HIGH	LOW
23	LOW	LOW	HIGH
24	LOW	LOW	LOW
24	LOW	LOW	LOW
26	LOW	LOW	LOW

27	LOW	LOW	LOW
28	LOW	LOW	HIGH
29	LOW	LOW	HIGH
30	LOW	HIGH	HIGH
31	LOW	LOW	LOW
32	LOW	HIGH	HIGH
33	LOW	HIGH	HIGH
34	LOW	HIGH	LOW
35	LOW	LOW	LOW
36	LOW	HIGH	LOW
37	LOW	LOW	LOW
38	LOW	HIGH	HIGH
39	LOW	HIGH	LOW
40	LOW	LOW	LOW
41	LOW	LOW	LOW
42	LOW	LOW	LOW
43	LOW	LOW	LOW
44	LOW	LOW	LOW
45	LOW	LOW	LOW
46	LOW	LOW	LOW
47	HIGH	HIGH	HIGH
48	LOW	HIGH	HIGH
49	LOW	HIGH	HIGH
50	HIGH	HIGH	HIGH
51	LOW	LOW	HIGH
52	LOW	HIGH	HIGH
53	LOW	HIGH	HIGH
54	LOW	HIGH	HIGH
55	LOW	LOW	LOW
56	LOW	LOW	LOW
57	LOW	LOW	LOW
58	LOW	HIGH	LOW
59	LOW	LOW	LOW
60	LOW	LOW	LOW
61	LOW	HIGH	LOW
62	LOW	HIGH	LOW
63	LOW	LOW	LOW
64	LOW	LOW	LOW
65	LOW	LOW	LOW

66	LOW	LOW	LOW
67	LOW	HIGH	LOW
68	LOW	LOW	LOW
69	LOW	LOW	LOW
70	LOW	LOW	LOW
71	LOW	LOW	LOW
72	LOW	LOW	LOW
73	LOW	LOW	LOW
74	LOW	LOW	LOW
75	LOW	HIGH	LOW
76	LOW	HIGH	LOW

Table 5: Expression profile of RASSF1a, APC1 and GSTP1 in the samples analyzed

High: ≥ 1 RQ value; Low: <1 RQ value.

Correlation of Gene Expression with Clinicopathological Parameters

The expression profiles of RASSF1a did not statistically correlate with any of the clinicopathological parameters analyzed despite the high percentage of downregulation of the gene. However, down regulation of APC1 correlated with the menopausal status with a p-value of 0.0082. GSPT1 downregulation statistically correlated with the FIGO stage of the samples analyzed (Table 6).

	Characteristics	**N**	**RASSF1A (silenced)**	**APC1 (silenced)**	**GSTP1 (silenced)**
Ovarian tumors		76			
Menopause State	Pre Menopause	25	24/25	17/25	16/25
	Post Menopause	51	49/51	32/51	38/51
	p-Value		p = 0.986837	**p= 0.00823608**	p = 0.342529
Histological type	Serous	43	40/43	28/43	26/43
	Mucinous	02	2/2	1/2	½
	Clear cell	04	4/4	3/4	2/4
	Endometriod	02	2/2	1/2	½
	p-Value		p = 0.898028	p = 0.90381	p = 0.959089
FIGO Stage	I-II	21	20/21	11/21	4/21
	III-IV	30	28/30	21/30	21/30
	p-Value		p = 0.776011	p = 0.200259	**p=0.000340569**
GRADE	**I-II**	17	16/17	11/17	7/17

	III-IV	34	32/34	22/34	23/34
	p-Value		P=1.0	P=1	p = 0.07019
CA125(U/ml)	0-35	14	14/14	9/14	11/14
	35-500	32	30/32	19/32	20/32
	500-1000	15	14/15	10/15	11/15
	>1000	15	15/15	11/15	11/15
	p-Value		p = 0.586679	p = 0.823271	p = 0.68326
Ascites	Presence of ascites	57	55/57	34/57	40/57
	Absence of ascites	19	18/19	13/19	11/19
	p-Value		p = 0.733771	p = 0.495454	p = 0.323785

Table 6: Gene expression profile and correlation with clinicopathological parameters and clinicopathological parameters.

Discussion

The silencing or downregulation of tumor suppressor genes can happen both via genetic and epigenetic alterations. Most common epigenetic alteration such as aberrant promoter hypermethylation of TSG such as RASSF1a, APC1 and GSTP1 are reported in ovarian cancers.

RASSF1a is a tumor suppressor gene that is known to play a role in regulating several cellular mechanisms ranging from microtubule stabilization to apoptosis. We have reported here that 100% of samples in the benign and 93.75% low malignant potential tumors show down regulation of RASSF1a indicating that the silencing of RASSF1a is indeed an early event in the initiation and progression of ovarian cancer. In addition 94.1% of the malignant samples also show down regulation of RASSF1a. A similar down regulation of RASSF1a gene is also reported in cancers such as colon and prostate [27,28].

We have observed that the APC1 gene is downregulated in 77.77% and 56.25% of benign and low malignant potential tumors and the down regulation of APC gene was found to be statistically correlated with menopausal status. Several studies have reported the silencing of APC1 in prostate, colon, breast and ovarian cancer [28,30].
GSTP1 expression analysis revealed a 100% down regulation in benign, an 81.25% down regulation in LMPs and the silencing of GSTP1 correlated significantly with FIGO stage. The down regulation of GSTP1 is reported to contribute to the tumorigenesis of prostate, endometrial, hepatocellular, bladder and ovarian cancers [31-37].

Furthermore we have observed that 37 of the 76 samples analyzed showed a down regulation of all the three TSGs, suggesting that the silencing of RASSF1a, APC1 and GSTP1 are playing a crucial role in tumor initiation.

Development of molecular mechanism for the diagnosis of cancers is emerging as a promising method for several cancers. Assessment of mRNA expression of tumor suppressor genes can serve as a promising tool in diagnosis and management of ovarian cancer. Identifying and understanding the molecular mechanism contributing to the inactivation of TSG will aid in designing effective strategy for the treatment and management of the disease. Reversal of the silencing of tumor suppressor genes could further prove to be an effective mechanism in inhibiting cancer growth and metastasis.

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Declarations

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Ethical Approval: The study was approved from the Institutional scientific and review board and medical ethics committee of Kidwai Memorial Institute of Oncology, Bangalore, India.

Conflict of Interest: The authors declare no conflict of interest.

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