Assessment of RASSF2A Gene Methylation in Ovarian Cancer Patients By Methylation – Specific Polymerase Chain Reaction

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Abstract: Members of the Rat sarcoma (Ras) associated domain family (RASSF) genes are thought to function as tumor suppressor genes (TSGs) by regulating the cell cycle and apoptosis. RASSF proteins consists of ten members termed from RASSF1 to RASSF10. RASSFs proteins are classified according to the location of Ras associated (RA) domain into either C-terminal (RASSF1-6) or N-terminal (RASSF7-10) family members. Ras associated domain Family member 2 (RASSF2) gene, also called rasfadin, is located on the short arm of chromosome 20 (20p13) and occupies 46 kilo base pairs of genomic DNA. RASSF2 gene encodes for three protein isoforms (RASSF2A, RASSF2B and RASSF2C). All of these isoforms have RA domain, but only RASSF2A gene encodes for a functioning protein and has a gene promoter rich cytosine phosphodinucleotides guanine (CpG) island. RASSF2A gene has 12 exons. RASSF2A gene transcription starts within a CpG island that spans exon 1 and 2**.** RASSF2A gene encodes for a functioning protein that has enzymatic activity to inhibit the growth function of Ras oncoproteins and has a gene promoter rich CpG island. RASSF2A gene is expressed in normal tissue and suppresses tumor growth. It is down regulated by promoter hypermethylation in CpG island. Inactivation of RASSF2A is detected in different tumors as colorectal, lung, gastric, breast and ovarian cancers. Our aim was to investigate the association of methylation as epigenetic change of RASSF2A gene and ovarian cancer. Epigenetics (A Greek expression for "above genetics") refers to reversible changes in gene expression without changes in gene structure. Our study was conducted on three groups. The first group was (16) sixteen newly diagnosed, untreated patients presented with malignant ovarian mass as diagnosed by ultrasound, histopathology and CA125 assay. The second group was (16) sixteen patients presented with benign ovarian mass as diagnosed by ultrasound, histopathology and CA125 assay. The third group was (16) sixteen apparently healthy women with matched age to the patients' groups. All these samples were tested to detect RASSF2A gene methylation using methylation specific PCR. In this study we found that 37.5% (6/16) of the malignant group were methylated and 62.5% (10/16) were unmethylated while in the benign group 6.3% (1/16) was methylated and 93.7% (15/16) were unmethylated and in healthy control group 0% (0/16) was methylated and 100% (16/16) were unmethylated. The results of the our study also revealed a statistical significant **(** $\chi 2 = 4.571$, P value = 0.033) increase in RASSF2A methylation in plasma of the malignant group more than the benign group, as RASSF2A methylation was detected in 37.5% (6/16) and 6.3% (1/16) was methylated and statistical significant ($\chi 2 = 7.385$, P value = 0.007) increase in RASSF2A methylation in plasma of the malignant group more than the control group, as RASSF2A methylation was detected in 37.5% (6/16) and 0% (0/16) was methylated. In conclusion, RASSF2A gene methylation was statistically higher in ovarian cancer cases than controls in Egyptian population. Therefore, it can be used as a potential non-invasive epigenetic marker for ovarian cancer. Thus our results provide the first insight into the contribution of RASSF2A gene methylation in ovarian cancer susceptibility in the Egyptian population.

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1. Introduction

Ovarian cancer (OC) is the leading cause of gynaecologic cancer death, while constituting only 3% of all female cancers worldwide ***(Hennessy et al., 2009).*** The poor survival rate of ovarian cancer patients is due to non-specific symptoms and the lack of sensitive and specific methods for the detection of early-stage ovarian cancer ***(Zahedi et al., 2012).***

Epithelial ovarian cancer is the most common ovarian malignancy with substantial histopathological heterogeneity ***(Koukoura et al., 2014).*** Serous epithelial OC is the most common subtype, with the majority of women presenting with advanced disease ***(Marsh et al., 2014).***

The widely used tumor biomarker for OC is CA125, which is a high molecular weight glycoprotein ***(Sarojini et al., 2012).*** It is elevated in some benign

conditions such as endometriosis and peritonitis and exhibit fluctuations associated with menstrual cycle and pregnancy ***(Moore et al., 2012).*** Despite of being used for current screening measures with trans-vaginal ultrasound, mortality rates remain high. As a result, no CA125 screening guidelines are recommended for the general population ***(Sarojini et al., 2012).***

It is recognized that both genetic and epigenetic events -refer to the changes in gene expression, with no changes in the DNA sequence- play a role in the development of ovarian cancer, with epigenetic changes occurring early in the carcinogenic process ***(Ting et al., 2013).***

DNA methylation comprises the best-known epigenetic mechanism associated with gene expression ***(Herman and Baylin, 2003).*** DNA methylation occurs on the cytosine residues of cytosine guanine dinucleotides (CG), also designated as (cytosine phosphodinucleotides guanine) CpG. Enzymes known as DNA methyltransferases (DNMTs) catalyse the addition of a methyl group to the cytosine ring to form methyl cytosine ***(Shaheen and Ahmed, 2013).***

The aberrant methylation of CpG island in gene promoters has been correlated with a loss of gene expression, and it appears that DNA methylation provides an alternative pathway to gene deletion or mutation for the loss of tumor suppressor gene (TSG) function ***(Baylin and Chen, 2005).***

The Ras association domain family (RASSF) genes are tumor suppressor genes (TSG), which encode RASSFs proteins that are negative Ras effectors proteins, which bind to active state of Ras proteins to inhibit cell growth and stimulate apoptosis ***(Fernandes et al., 2013).*** The RASSF2 gene, which is located on chromosome 20, encodes for three protein isoforms (RASSF2A, RASSF2B and RASSF2C) ***(Hesson and Latif, 2010).*** RASSF2A gene contains CpG Island in its promoter and it is reported to be inactivated by its promoter methylation in several human cancers ***(Zhang et al., 2006).***

Due to the high mortality rate associated with ovarian cancer, a number of studies have been carried out in an attempt to discover novel therapeutic approaches ***(Vaughan etal., 2011).***

Therefore, a study on the molecular mechanism underlying ovarian cancer progression, including a search for methylation status, is important for early diagnosis and effective therapy for ovarian cancer. There are limited data about the genetic cause in ovarian cancer ***(Wu et al., 2014).***

2. Materials and methods:

Study subjects:

The study was conducted in Clinical Pathology Department, Clinical Chemistry Unit, Ain Shams University Hospitals. The study population was recruited from Gynaecology and Obstetrics departments of Ain Shams University Hospitals (Al-Demerdash and Ain Shams Specialized Hospitals) from May 2015 to January 2016 after verbal consent. Inclusion criteria for the studied cases included any patient with newly diagnosed, untreated ovarian mass diagnosed by ultrasound and histopathology. Exclusion criteria were infection with hepatitis virus B or C, pregnancy and patients diagnosed as polycystic ovarian syndrome. Three groups were selected in this study. The first group was (16) sixteen newly diagnosed, untreated patients presented with malignant ovarian mass as diagnosed by ultrasound, histopathology and CA125 assay. The second group was (16) sixteen patients presented with benign ovarian mass as diagnosed by ultrasound, histopathology and CA125 assay. The third group was (16) sixteen apparently healthy women with matched age to the patients' groups.

Seven millilitres (7 mL) of venous blood were withdrawn from all subjects included in the study under complete aseptic conditions. The collected blood was withdrawn on EDTA tubes for PCR and a plain test tube for CA125 assessment. Two millilitres (2 mL) plasma was separated by centrifugation at 3000 rpm for 10 minutes and were divided into 2 aliquots to be stored at -20 until RASSF2A gene methylation analysis. Hemolysed samples were discarded, also repeated freezing and thawing was avoided.

All individuals included in the study were subjected to full history taking with emphasis on family history of ovarian and breast cancer, general clinical examination and abdominal examination for adnexal mass and histopathalogical biopsy for both benign and malignant patients' groups. Serum CA125 was measured by ELISA for patients' group only and RASSF2A gene methylation was measured by conventional MSP for all groups.

Genotyping analysis:

Detection of RASSF2A gene methylation by MSP: **Step 1:** Genomic DNA extraction: DNA was extracted from plasma samples by QIAamp DNA blood Midi kit (QIAGEN Incorporation, 28159 Avenue, Stanford Valencia, CA91355, USA, USA, Cat 51185) according to the manufacturers' instructions. The extraction kit supplies QIAGEN protease and lysis buffer (AL) for sample lysis. The lysate is loaded into QIAamp spin column so the DNA is adsorbed onto the silica membrane. Several wash steps by wash buffers (AW1 and AW2). Finally the DNA is eluted using the elution buffer (AE) provided with the kit.A 200µL QIAGEN Protease was added to 2 mL plasma in a 15 mL centrifuge tube and mixed briefly by pipetting then a 2.4 mL lysis buffer (AL) is added to the mixture and mixed by inversion for 15

times, followed by additional vigorous shaking for at least 1 minute. The whole solution was incubated at 70°C for 10 minutes to reach the maximum lysis. Two mL ethanol conc. (96-100%) was added then followed by vigorous shaking.

The whole solution was then transferred onto QIAamp Midi column placed into 15 mL centrifuge tube then centrifuged at 3000 rpm for 3 minutes after which the filtrate was discarded.

Two mL of wash buffer (AW1) was added to the Midi column followed by centrifuge at 5000 rpm for 1 minute then 2 mL wash buffer (AW2) was added to Midi column and centrifuged at 5000 rpm for 15 minutes to remove the residual contaminants. The collecting tubes containing the filtrate were discarded and the Midi column was placed in a new clean 15 mL centrifuge tubes. Finally, elution was done by adding300µL elution buffer (AE) onto the membrane of the Midi column and incubated for5 minutes in the room temperature then centrifuged at 5000 rpm for 2 minutes. The filtrate was stored at -20 until time of bisulfite modification steps and DNA amplification.

Step 2: Bisulfite modification:

Bisulfite modification for extracted DNA was done using Epi Tect Fast Bisulfite Conversion kit (QIAGEN, USA, Catalogue 59824). Bisulfite treatment of target DNA with sodium bisulfite results in complete conversion of unmethylated cytosine into uracil while leaving methylated cytosine unchanged giving rise to different DNA sequence. Bisulfite conversion is followed by removal of bisulfite salts and chemicals used in the bisulfite modification process. The bisulfite mixture is placed in thermal cycler for complete conversion of DNA.

The whole mixture was then transferred to Min-Elute DNA spin column followed by centrifugation at maximum speed (12000 rpm) for 1 minute then the flow-through is discarded. 500µL of the wash buffer (BW) was added to each spin column for efficient removal of residuals of bisulfite mixture, followed by centrifugation at maximum speed for one minute, and then the filtrate was discarded. 500µL of Desulfonation buffer (BD) was added followed by incubation of samples in room temperature for 15 minutes then centrifugation at maximum speed for 1 minute and then filtrate was discarded. Another 500µL of the wash buffer (BW) was added followed by centrifugation for 1 minute at maximum speed and finally the filtrate were discarded. A 250µL ethanol was added to each sample followed by centrifugation at maximum speed for 1 minute then spin columns are placed into new 2 mL collection tubes.

The spin columns were placed into clean 1.5 mL microcentrifuge tubes and 20µL elution buffer (EB) was added to each sample, then samples were incubated for 1 minute in the room temperature followed by centrifugation at maximum speed for 1 minute to get the elute that can be stored at -20 °C until amplification.

EpiTect MSP (QIAGEN, USA, Cat 59305) and primers that were supplied from (*Invitrogen Corporation, 1600 Faraday Ave, Carlsbad, CA 92008, Canad*a) were used for DNA amplification. The MSP master mix contains HotStar Taq d-Tect Polymerase that represents a modified form of polymerase, which helps discrimination of single base, mismatching during primer annealing and extension. Polymerase is present in assay buffer of balanced combination of KCl and $(NH4)_2SO4$ and MgCl and 200µM of each deoxynucleoside triphosphates (dNTP).

For all patients and control samples, reaction mixtures were preformed in duplicates, one with methylated set of primers and the other with unmethylated primers and samples were placed in the thermal cycle. In each run, a negative control tube was included to exclude contamination. It contained water instead of DNA template.

Step 4: DNA detection by gel electrophoresis:

Amplified DNA product was loaded on 2% high-resolution agarose gel stained with ethidium bromide and separated by electrophoresis (100V for 30 minutes). A ladder was used to mark the bands. Ultraviolet transilluminator was used to visualize the separated bands.108 bp bands were detected for both methylated and unmethylated samples.

Statistical analysis:

Statistical analysis was done using software version (V. 23.0, IBM Corp., USA, 2015) of SPSS (Statistical package for the social sciences). The methods used for statistical analysis were as follows:

i. Descriptive Statistics:

a. The median and inter-quartile range (IQR): values of the skewed parameters as age and CA125 level are expressed as median and IQR (25th – 75th).

ii. Significance Tests:

b. Wilcoxon Rank Sum test: Compares between two independent groups for non-parametric data as for age and CA125 level.

c. Chi-square test (X^2):

Study the association between each 2 variables or comparies between 2 independent groups regarding the categorized data. The probability of error at 0.05 (*p* value <0.05) was considered significant while at 0.01 and 0.001 are highly significant. *P* values were calculated by two-sided test; the probability (*P*) value for the calculated $\chi 2$ value was then deduced, with the degrees of freedom being equal to the sum of the two sample sizes minus 2. *P* values > 0.05 indicate a non-significant difference, $P < 0.05$ indicates a significant difference for all statistical analyses ***(Fogiel, 1986).***

Table (1): Primer sequence of RASSF2A***(Zhang et al., 2006)***

Sequences of methylated RASSF2A gene	**Forward primer (F):** 5′ATTCGTCGTCGTTTTTTAGGCG3′ **Reverse primer (R):** 5′AAAAACCAACGACCCCCGCGG3′
Sequences of unmethylated RASSF2A gene	**Forward primer (F):** 5′AATTTGTTGTTGTTTTTTAGGTGG3′ **Reverse primer (R):** 5′-AAAAAACCAACAACCCCCACA -3′

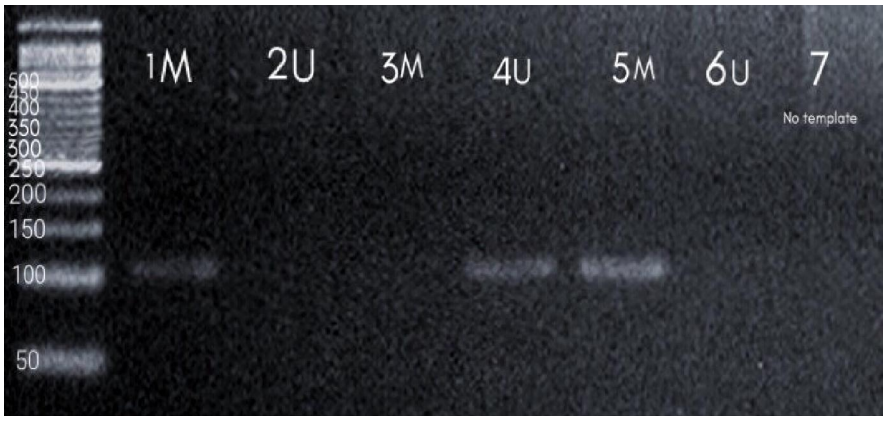

Figure (1): Gel electrophoresis for RASSF2A gene methylation

From left to right:
A50 bp ladder was used as molecular weight marker
Lane (1) and (2): Samples from patient with malignant OC show methylated band
Lane (3) and (4): Samples from patient with malignant OC show an unmethylated band
Lane (5) and (6): Samples from patient with malignant OC show methylated band
Lane (7): A water control as a negative control to detect contamination in PCR reaction

3. Results

A total number of 16 patients with malignant ovarian cancer (OC), 16 patients with benign ovarian mass and 16 healthy controls were enrolled in the study.

The descriptive statistics of the studied groups of malignant OC (group I), benign ovarian mass group (group II) and the healthy control group (group III) are shown in **table 2.** The data showed non-parametric distribution so the median and the interquartile range (IQR; 25th percentile to 75th percentile) were used. The median age of the studied malignant OC group was 43 years (IQR: 37-52) and was 39.5 years (IQR: 30-48) for the studied benign group, while that of the healthy controls was 40 years (IQR: 30-42). The median age of menopause in the studied malignant group was 48 years (IQR: 47-51.7) and that of the benign group was 52 years (IQR: 49-52) while none of the participant in the healthy controls had menopause. The median age of the first pregnancy was 24 years (IQR: 20-27) in the studied malignant group and was 23 years with (IQR: 22-30) in the benign group, while in the healthy control group was 24 years (IQR: 22-28).

The CA125 median level was 334 IU/mL (IQR: 124-1461) in the malignant group and 14 IU/mL (IQR: 11-26) in the benign group. CA125 level was not measured in the healthy controls according to our protocol of thesis.

Table 3 shows that 62.5% (10/16) in the malignant group were ≥ 50 years while 81.3% (13/16) were ≥ 50 years in the benign group. As regards the menopausal status, 50% (8/16) of the malignant group were in the menopausal status, while in the benign group 18.7% (3/16) were in the menopausal status. Regarding parity, 6.3% (1/16) of the malignant group were nullipara, while 18.7% (3/16) in the benign group. Positive family history of OC was equally seen in both the malignant and the benign groups as 6.3% (1/16) was positive for family history. CA125 level of 87.5% (14/16) of the malignant group and 6.3% (1/16) of the benign group were >35 IU/mL.

There was no statistically significant difference ($P > 0.05$) as regards age, age of menopause and age of the 1st pregnancy by Wilcoxan Rank Sum Test between the benign and the malignant groups as shown in **table 3** while CA125 serum levels were significantly higher in the malignant OC group compared to the benign group ($p < 0.01$).

The descriptive statistics of RASSF2A methylation status in the three groups of the study are shown in **table 4** and **figure 2** and show 37.5% (6/16) of the malignant group were methylated and 62.5% (10/16) were unmethylated while in the benign group 6.3% (1/16) was methylated and 93.7% (15/16) were unmethylated and in healthy control group 0% (0/16) was methylated and 100% (16/16) were unmethylated.

The descriptive statistics of the demographic and laboratory data among RASSF2A gene methylated group versus unmethylated group in malignant OC patients are shown in **table 5** and the data showed non-parametric distribution so the median and the interquartile range were used. The median age of methylated group of the malignant OC patients was 36 years (IQR: 27-57.7) and 53 years (IQR: 47.3-66.5) in the unmethylated group of OC patients. The median age of menopause of the methylated OC group was 49.5 years (IQR: 47-52), and 48years in the unmethylated group. Regarding the age of 1st pregnancy, the median age was 23 years (IQR: 19-26) in the methylated OC group and 23.5 years (IQR: 20.5-27.5) in the unmethyalted. The CA125 median was 133 IU/mL (IQR: 57-867) in methylated OC group and 870 IU/mL (IQR: 124.8-1619.8) in unmethylated group.

There was no statistically significant difference ($p > 0.05$) as regard age, menopausal age, age of 1st pregnancy and CA125 serum level between the methylated and the unmethylated groups by Wilcoxan Rank Sum Test as shown in **table 5.**

The comparative statistics among RASSF2A methylated group versus unmethylated group in OC patients as shown in **table 5** using the following data**;** age, family history of breast cancer, family history of OC, metastasis, ascites, abdominal pain, parity and show no statistically significance except for ascites ($p <0.05$) by Chi-square test.

The descriptive and comparative statistics between the groups of the study and methylation status are shown in **tables 6, 7** and **8** and show no statistically significance differences between group II and III ($p >0.05$) in **table 7**, a statistical significance ($p <0.05$) is found between group I and II in **table 6** while **table 8** shows highly statistical significance ($p <0.01$) between group I and III by using Chi-square test.

Table (2): Descriptive statistics of the demographic and laboratory data of the three studied groups

Group / Parameter	Group I Malignant (n=16)	Group II Benign (n=16)	Group III Control (n=16)
Age/years			
Median	43	39.5	40
IQR	37-52	30-48	30-42
Age of menopause/years			
Median	48	52	--
IQR	47-51.7	49-52	--
Age of 1st pregnancy/years			
Median	24	23	24
IQR	20-27	22-30	22-28
CA125 (IU/mL)			
Median	334	14	Not performed acc. to protocol of thesis
IQR	124-1461	11-26	

Interquartile range (IQR) = first quartile (Q_1) - third quartile (Q_3).

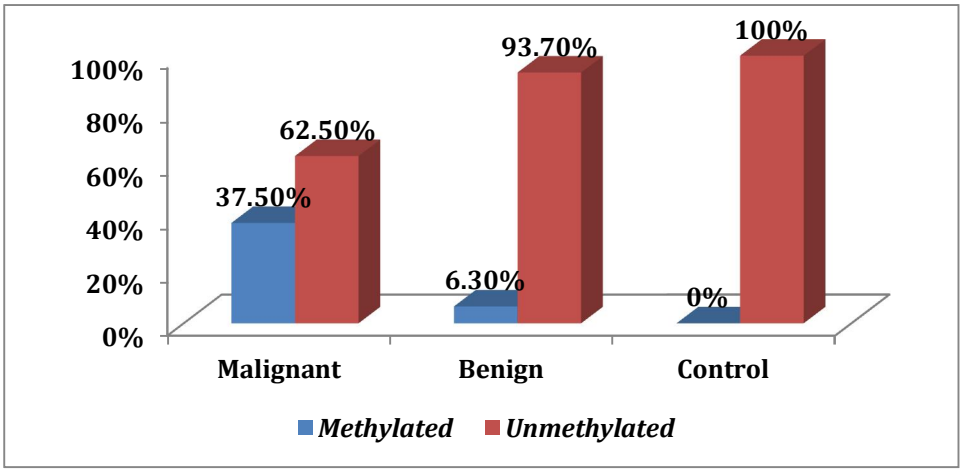

Figure (2): Comparison between the three studied groups and methylation status

Table (3): Descriptive and comparative statistics of demographic and laboratory data between group I (malignant) and group II (benign) using Wilcoxan Rank Sum Test

Group / Parameter	Group I Malignant (n=16)	Group II Benign (n=16)	z	*p*	Sig.
Age/years					
Median	43	39.5	-1.094	0.274	NS
IQR	37-52	30-48			
Menopausal age/years					
Median	48	52	-1.242	0.214	NS
IQR	47-51.7	49-52			
Age of 1st pregnancy/years					
Median	24	23	-0.659	0.51	NS
IQR	20-27	22-30			
CA125 Levels (IU/mL)					
Median	334	14	**-4.353**	**0.0**	**HS**
IQR	124-1461	11-26			

Table (4): Descriptive statistics of RASSF2A methylation status in the three studied groups

Group / Parameter	Group I Malignant (n=16)	Group II Benign (n=16)	Group III Control (n=16)
RASSF2A methylation status			
Methylated	n=6 (37.5%)	n=1 (6.3%)	n=0 (0%)
Unmethylated	n=10 (62.5%)	n=15 (93.7%)	n=16 (100%)

Table (5): Descriptive and comparative statistics of the demographic and laboratory data among RASSF2A gene methylated group versus unmethylated group in malignant OC patients using Chi-square test

Group / Item	Methylated (n=6)	Unmethylated (n=10)	Chi –square value	*p* value	Sig.
Age ≥ 50/years	n=2 (33.3%)	n=8 (80%)	3.4844	0.06	NS
Positive for family history of BC	n=1 (16.7%)	n=1 (10%)	0.152	0.696	NS
Positive for family history of OC	n=1 (16.7%)	n=0 (0%)	1.778	0.182	NS
Positive for metastasis	n=3 (50%)	n=3 (30%)	0.640	0.424	NS
Positive for ascites	n=1 (16.7%)	n=8 (80%)	**6.112**	**0.013**	**S**
Presented with Abdominal pain	n=2 (33.3%)	n=4 (40%)	8.178	0.317	NS
Having a child or more	n=6 (100%)	n=9 (90%)	2.286	0.319	NS

Table (6): Descriptive and comparative statistics between group I and group II regarding RASSF2A gene methylation using Chi-square test

Group / Item	Group I Malignant (n=16)	Group II Benign (n=16)	Chi-square value	*p* value	Sig.
Methylated	n=6 (37.5%)	n=1 (6.3%)	**4.571**	**0.033**	**S**
Unmethylated	n=10 (62.5%)	n=15 (93.8%)			

Table (7): Descriptive and comparative between group II and group III regarding RASSF2A gene methylation using Chi-square test

Group / Item	Group II Benign (n=16)	Group III Control (n=16)	Chi-square value	*p* value	Sig.
Methylated	n=1 (6.3%)	n=0 (0%)	1.032	0.310	NS
Unmethylated	n=15 (93.8%)	n=16 (100%)			

Table (8): Descriptive and comparative statistics between group I and group III using Chi-square test regarding RASSF2A gene methylation

Item	Group I Malignant (n=16)	Group III Control (n=16)	Chi-square value	*p* value	Sig.
Methylation	n=6 (37.5%)	n=0 (0%)	**7.385**	**0.007**	**HS**
Unmethylation	n=10 (62.5%)	n=16 (100%)			

4. Discussion

Ovarian cancer is the leading cause of gynaecologic cancer death, while constituting only 3% of all female cancers worldwide ***(Hennessy et al., 2009).*** The poor survival rate of ovarian cancer patients is due to non-specific symptoms and the lack of sensitive and specific methods for the detection of early-stage ovarian cancer ***(Zahedi et al., 2012).***

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Thus RASSF2A gene methylation was statistically higher in ovarian cancer cases than controls in Egyptian population. Therefore, it can be used as a potential non-invasive epigenetic marker for ovarian cancer. Thus our results provide the first insight into the contribution of RASSF2A gene methylation in ovarian cancer susceptibility in the Egyptian population.

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