Association of SNPs in miR-196a2 with the risk of Ovarian Cancer and its relation with Clinicopathological data

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Abstract: Objective: The rs11614913 polymorphism of miR-196a2 is associated with carcinogenesis in several types of cancer. This study aims to investigate the association between miR-196a-2 (rs11614913) SNP and ovarian cancer risk in a group of Egyptian patients. **Methods:** This retrospective study included 50 newly-diagnosed patients with different stages of ovarian cancer, 15 patients with benign ovarian tumors, and 35 healthy femalevolunteers as a control group. Venous blood samples were collected to detect serum CA-125 and serum HE4 in addition to DNA extraction and detection of miR-196a2 (rs11614913) SNP. **Results:** Ovarian cancers were mostly epithelial with advanced stage. Ovarian cancer group had significantly higher frequencies of CT and TT genotypes compared to controls, andbenign ovarian groups separated (p = 0.037) or combined (p = 0.008). The presence of CT genotype is associated with a risk of developing ovarian cancer with an OR of 3.9 (95%CI: 1.5-10.2). The TT genotype was associated with a risk of developing ovarian cancer with an OR of 3.8 (95%CI: 1.3-10.9). The T allele was associated with a risk of developing ovarian cancer with an OR of 2.4 (95%CI: 1.3-4.2). CT/TT genotypes were significantly associated with advanced stage (p = 0.004) and presence of metastasis (p < 0.001), but not associated with grade (p = 0.629), or levels of tumor markers. **Conclusion:** ThemiR-196a-2 rs11614913 polymorphism may serve as a diagnostic and prognostic biomarker in ovarian cancer.

Keywords: Association; SNPs; miR-196a2; risk; Ovarian; Cancer; relation; Clinicopathological data

1. Introduction:

Ovarian cancer accounts for about 4% of all cancer cases in women. But, it is one of the major causes of cancer-related death in females.[1]The risk factors for ovarian cancer include early menarche, late menopause, family history, long-term use of ovulation-inducing drugs, carriers of mutated BRCA1/2genes, and other genetic factors.[2] Epithelial ovarian cancer (EOC) accounts for 90% of all ovarian cancer. They are classifiedinto serous, mucinous, endometrioid, and non-clear cell types.[3]

Survival rates of early-stage ovariancancer are high; the 5-year survival rate exceeds 90 %. Nonetheless, in patients with stage III or IV ovarian cancer, the 5-year survival rate is as poor as 30%.[4]This poor prognosis of ovarian cancer is attributed to the insidious symptomatic nature in early stage and lack of a reliable and minimally invasivemethod for early detection.[5]Moreover, EOC has an accelerated and aggressive growth pattern, which may lead to high recurrence rates.[6]Therefore, innovativeapproaches for the detectionof early-stage ovarian cancer are mandatory for proper and timely management.

MicroRNAs (miRNAs) are a class of naturally occurring, small noncodingRNA molecules with complex biological functions. MicroRNAsregulate the expression of numerous proteins through promoting messenger RNA (mRNA) degradation, inhibiting mRNA translation, and affecting transcription by binding to the 3'-untranslated region of their target mRNA.[7]

It has been shownthat miRNAs are involved in ovarian cancer tumorigenesisthrough malignant transformation, differentiation, proliferation, and apoptosis.[8,9] Also, single nucleotide polymorphisms (SNPs) or mutations in the miRNA genes may influence the property and expression of the miRNAs.[10]The rs11614913 polymorphism of miR-196a2 has been found to affect itsexpression and is associated with carcinogenesisin several types of cancer.[11–13]

This study aims to investigate the association between miR-196a-2 (rs11614913) SNP and ovarian cancer risk in a group of Egyptian patients and whether there is an association between this polymorphism and clinicopathological characteristics in these patients.

2. Patients and Methods

Patients:

This retrospective study was performed at the National Cancer Institute; Cairo University, Egypt during the period from September 2015 to August 2017 on 100 female subjects; 50 newly-diagnosed patients with different stages of ovarian cancer, 15 patients with benign ovarian tumors, and 35 healthy female volunteers with comparable age as a control group. Ovarian histopathological diagnoses and clinical stages were classified according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. [14] Exclusion criteria of ovarian cancer patients are pregnant or lactating women and serious medical or psychiatric illness. Blood samples were obtained from patients before any treatment. Clinical and laboratory data were collected from patients' files.

Methods:

Five milliliters of venous blood samples were collected. Two milliliters were put on serum vacutainer tubes, centrifuged at 3000 rpm for 15 minutes at room temperature to obtain serum for detection of serum CA-125 and serum HE4 using the full automatic chemiluminescence analyzer (Cobas e400) and the corresponding kit according to manufacturer protocol (Roche, Diagnostic, Indianapolis, IN, USA). Three milliliters of venous blood samples were put on EDTA vacutainer tubes, centrifuged at 3000 rpm for 15 minutes at room temperature to obtain the buffy coat, a leukocyte-enriched fraction of the whole blood. They were stored at -20°C till the time of DNA extraction and subsequent detection to SNPs in miR-196a2.

Extraction of DNA from peripheral blood:

DNA extraction was done using a QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen Catalog no.51104). DNA was isolated from 200 µL of buffy coat and was eluted in buffer AE and then stored at -20°C till amplification. DNA samples were measured using Nanodrop 1000 to detect their concentrations (Thermo Scientific, Wilmington, DE, USA).

DNA amplification

This was done in the thermal cycler the reaction setting was as follows: incubation at 37°C for 60 min followed by 5 min at 95°C. The cDNA was stored at −20°C until use.[15]

Detection of miR-196a2 (rs11614913) SNP

It was done using Applied Biosystems Step-One™ TaqMan Real-Time PCR System Assay (Catalog no. 43763542). The thermal cycling program conditions were done according to the manufacturer's instructions, the cycling conditions were as follows (Table 1):

Table 1: Thermal cycling condition for probe optimization

	Pre-PCR read	**AmpliTaq Gold Enzyme activation**	**PCR (45 cycles)**	
	Step 1 *Hold*	Step 2 *Hold*	Step 3 *Denaturate*	Step 4 *Anneal/Extend*
Temperature	60˚C	95˚C	92˚C	60˚C
Time	30 sec	10 min	15 sec	1 min
Volume	25 µl			

TaqMan Genotyping Assay mix: (C_15946934_10)

The Genotyping Assay was supplied in a tube containing sequence-specific forward and reverse primers to amplify the polymorphic sequence.

Two TaqMan® MGB probes:

- One probe labeled with VIC® dye detects the Allele 1 sequence.

- One probe labeled with FAM™ dye detects the Allele 2 sequence.

Allele 1 is the normal or wild-type while Allele 2 is the abnormal or mutant type. A minor groove binder (MGB) is a modification at the 3′ end of each probe which increases the melting temperature (Tm) for a given probe length allowing the design of shorter probes. Shorter probes result in greater differences in Tm values between matched and mismatched probes, producing robust allelic discrimination.[16]

Interpretation of results

After PCR amplification, an endpoint plate read using an Applied Biosystems Real-Time PCR System was done. The Sequence Detection System (SDS) Software uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample.

Allele 1/Allele 1 = wild or normal type (CC).

Allele 2/ Allele 2 = mutant or abnormal type (TT).

Allele 1/ Allele 2 = heterozygous type (TC).[17]

Statistical methods:

Statistical analysis was done using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). Numerical data were expressed as a mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's

exact test) was used to examine the relationship between qualitative variables. For quantitative data, the comparison between two groups was done using independent sample t-test or Mann-Whitney test. Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Logistic regression was used to estimate the risk of genotypes expressing the risk as odds ratio (OR) with it 95% confidence interval (CI). The Receiver Operating Characteristic (ROC) curve was used for prediction of cut off values. A p-value < 0.05 was considered significant.

3. Results

The most frequent pathological type of ovarian cancer was the serous adenocarcinoma (62%) (Representative examples of histopathologic types are presented in figure 1). The majority of the ovarian cancer group has metastasis, either distant or peritoneal. Therefore, the majority had advanced stage (Table 2).
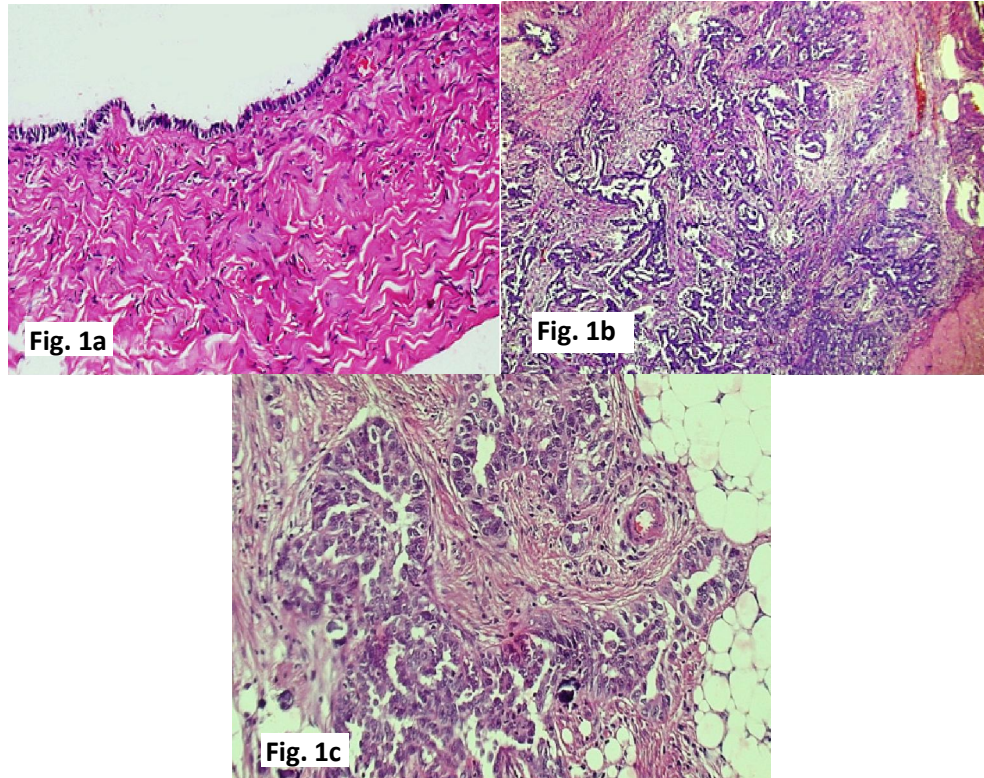

Figure 1: Representative examples of studied ovarian tumors (hematoxylin and eosin). 1a: a case of benign serous cystadenoma (Mag 20x). 1b: a case of serous adenocarcinoma (Mag 10x) with positive peritoneal metastasis (fig 1c) (Mag 20x)

Table 2: Histopathologic and clinical characteristics of ovarian cancer and benign ovarian groups

	Number	Percentage
Ovarian Cancer (n=50)		
Histological Types		
Serous adenocarcinoma	31	62.0
Mucinous adenocarcinoma	5	10.0
Endometrioid adenocarcinoma	3	6.0
Others	11	22.0
Metastasis		
Non-metastatic	11	22.0
Peritoneal Metastasis	20	40.0
Distant Metastasis	19	38.0
Grade		
I	6	12.0

	Number	Percentage
II	26	52.0
III	18	36.0
FIGO stage		
IA	3	6.0
IIA	3	6.0
IIC	4	8.0
IIIB	3	6.0
IIIC	19	38.0
IV	18	36.0
Positive **Family history**	2	4.0
Benign Ovarian Tumors (n=15)		
Benign serous cystadenoma	10	66.7
Others	5	33.3

Table 3: Age and tumor marker levels in the three studied groups

	Ovarian Cancer Group n=50	**Benign Group n=15**	**Control Group n=35**	**p value**
Age (years)	44.1±11.4[a]	41.1±11.3[ab]	37.9±9.5[b]	0.040
CA125	203.5 (5.3-4618.0)[a]	22.6 (0.2-201.0)b	11.0 (4.0-30.0)[b]	< 0.001
HE4	959.5 (432.0-6543.0)[a]	80.0 (55.0-160.0)[b]	16.0 (5.0-67.0)[c]	< 0.001

Ovarian cancer group was significantly older than the control group ($p = 0.034$). CA125 was significantly higher in ovarian cancer group compared to the control group ($p < 0.001$) and benign ovarian group ($p = 0.004$). The three groups had significantly different HE4 levels relative to each other (Table 3).

Table 4: miRNA-196a-2 gene polymorphism in the three studied groups

	Ovarian Cancer Group n=50	**Benign Group n=15**	**Control Group n=35**
CC	11 (22.0%)	8 (53.3%)	18 (51.4%)
CT	23 (46.0%)	5 (33.3%)	9 (25.7%)
TT	16 (32.0%)	2 (13.3%)	8 (22.9%)

The genetic frequencies of miR-196a2 are described in Table 4. Thegenotype distribution of miR-196a2 in ovarian cancer group was in agreement with HWE ($p = 0.617$). But, the genotype distributions of controls and all studied cases was not consistent with HWE ($p = 0.007$, and $p = 0.012$, respectively). Ovarian cancer group had significantly higher frequencies of CT and TT genotypes compared to controls, andbenign ovarian groupsseparated ($p = 0.037$) or combined ($p = 0.008$). Using logistic regression analysis, the presence of CT genotype is associated with a risk of developing ovarian cancer with an OR of 3.9 (95%CI: 1.5-10.2). The TT genotype was associated with a risk of developing ovarian cancer with an OR of 3.8 (95%CI: 1.3-10.9). The T allele was associated with a risk of developing ovarian cancer with an OR of 2.4 (95%CI: 1.3-4.2).

Table 5: miRNA-196a-2 genotypes and allele frequency in ovarian cancer group versus combined benign and control groups

	Ovarian Cancer Group n=50	**Benign + Control n=50**	**p value**
Genotype			
CC	11 (22.0%)	26 (52.0%)	0.008
CT	23 (46.0%)	14 (28.0%)	
TT	16 (32.0%)	10 (20.0%)	
Alleles			
C	45%	66%	0.003
T	55%	34%	

Table 6: Relation between miRNA-196a-2 genotypes and clinicopathological characteristics of cancer cases

	CC n=11	CT/TT n=39	p value
Stage			
I/II	6 (60.0%)	4 (40.0%)	0.004
III/IV	5 (12.5%)	35 (87.5%)	
Grade			
I	2 (33.3%)	4 (66.7%)	0.629
II	6 (23.1%)	20 (76.9%)	
III	3 (16.7%)	15 (83.3%)	
Metastasis			
Non-metastatic	8 (72.7%)	3 (27.3%)	< 0.001
Metastasis	3 (7.7%)	36 (92.3%)	
CA125	80 (19-3011)	300 (5-4618)	0.194
HE4	898 (453-3210)	1234 (432-6543)	0.174

CT/TT genotypes were significantly associated with advanced stage (p = 0.004) and presence of metastasis (p < 0.001), but not associated with grade (p = 0.629), or levels of tumor markers (Table 6).

4. Discussion:

This study demonstrated significantly higher frequencies of CT and TT genotypes of miR-196a-2 rs11614913 polymorphism in ovarian cancer group compared to controls and benign ovarian groups whether separately (p = 0.037) or combined (p = 0.008). The heterogeneous CT genotype and homogenous TT genotype were associated with 3.9-fold and 3.8-fold risk of ovarian cancer, respectively. The T allele was associated with 2.4 increased risk of ovarian cancer. The rs11614913 polymorphism might have a prognostic significance in ovarian cancer as the CT/TT genotypes were associated with advanced stage and metastatic disease.

MicroRNAs could be used as biomarkers in human cancers, including gastric cancer, lung cancer, prostate cancer, breast cancer and others.[18–21]It has been shown that miR-196a plays a role incancer genes in many cancers but with different relative signal mechanism. For example, in pancreatic cancer, high expression of miR-196a was reported. Downregulating the expression of miR-196aincreased the expression of NFKBIA protein.[22]A recent study showed that the relative expression quantities of miR-196a in ovarian cancer tissue were significantly higher than in normal ovarian epithelial tissue and in benign ovarian tissue. The authors indicated that poorly differentiated cancer cells had higher expression quantity of miR-196a.[23]

Bioinformatics found that 3′-UTR HOXA10 mRNA possessed a specific binding sequence of miR-196a and it is one of the downstream targets of miR-196a.[23] It has been shown that overexpression of HOXA10 was associated with the occurrence of ovarian cancer.[24,25] Yang et al.[23] found a significant decrease of the expression quantity of HOXA10 after down-regulation of the expression of miR-196a. These findings indicated that miR-196a might act as a cancer-promoting gene that promotes the migration and invasion of epithelial ovarian cancer by downstream target gene HOXA10.[23]

Previous studies reported contradictory results about the role of the miR-196a-2 rs11614913 polymorphism in different cancers including ovarian cancer. A Turkish study showed that the CC genotype of the miR-196a-2 rs11614913 polymorphism is associated with increased risk of HCC development.[26] Another study found significantly increased risk of gastric cancer in association with the homozygote variant CC of miR-196a-2 compared with homozygote TT and heterozygote CT carriers.[27] Similarly, the CC genotype was more frequently encountered in colorectal cancer patients than controls in a Chinese population.[28] A meta-analysis of 11 studies of the association between miR-196a2 polymorphisms and cancer risk suggested that rs11614913 probably contributes to decreased susceptibility to cancer, especially in Asians and breast cancer.[29]Another meta-analysis suggested that the allele frequency and the genotype distribution of miR-196a2were not associated with gastric cancer risk in the five genetic models.[30]

Song et al.[31] studied the distribution frequencies of miR-196a-2 rs11614913 in a group of Chinese population havingepithelialovarian cancer. In contrast to the present study, the CC genotype was associated with a 1.34-fold increase in ovarian cancer risk. In another series of Chinese patients with ovarian cancer, there was no significant relationship between the miR-196a2 polymorphism andovarian cancer risk in all models.[32] In a third Chinese series, the frequencies of CT genotype, TT, and TT/CT genotypes were found to be significantly higher in cancer cases than in controls. The OR values for

higher susceptibility to ovarian cancer of TTand CT were 3.0 and 3.7, respectively.

We can conclude that miR-196a-2 rs11614913 polymorphism may serve as a diagnostic and prognostic biomarker in ovarian cancer. The CT and TT genotypes are more frequent in ovarian cancer patients compared to controls. These two genotypes were associated with nearly 4-fold increased risk of ovarian cancer. The T allele was associated with 2.4 increased risk of ovarian cancer.

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