

Association between *miR-196a-2* Gene Polymorphism and Ovarian Cancer Prognosis in Egyptian Females

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Abstract

Background: Ovarian cancer is the fifth leading cause of cancer-related deaths among women worldwide. Unfortunately, early detection tests are relatively lacking. Diagnosis in the late stages of the disease carries a poor prognosis. **Objective:** To evaluate the relationship between *miR-196a-2* rs11614913 polymorphism and ovarian cancer risk and prognosis in Egyptian females. **Methods:** In this case-control study, the participants were classified into 2 groups. Group A is the control group which included 50 healthy females. Group B included 50 patients newly diagnosed with ovarian carcinoma confirmed by histopathological analysis. Immunohistochemistry for P53 and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for *miR-196a-2* genotypes detection were performed. **Results:** There was a statistically significant difference among ovarian cancer cases and controls regarding genotypes ($P = 0.003$). However, the distribution of the T and C alleles in both studied groups showed no significant difference ($P = 0.17$). There was a statistically significant increase of CA 125 levels among CT and CC genotypes carriers of ovarian cancer cases ($p = 0.04$). Besides, there was a statistically significant correlation between *miR-196a-2* polymorphism and each of tumor grade ($P < 0.001$), p53 immunohistochemical expression ($P = 0.002$), and Figo classification ($P < 0.001$). **Conclusion:** There was a statistically significant increase of CA 125 levels among C allele carriers of ovarian cancer cases. Besides, there was a statistically significant association between the *miR-196a-2* polymorphism and each of tumor grade, p53 immunohistochemical expression, and Figo classification. So, *miR-196a-2* polymorphism can be a possible prognostic factor in ovarian cancer.

Keywords: miR-196a-2- polymorphism- ovarian cancer- P53

Asian Pac J Cancer Prev, **23** (5), 1761-1768

Introduction

MicroRNAs (miRNAs) are 18–25 nucleotide-long, single-stranded noncoding RNA that play an important role in the regulation of mammalian gene expression via post-transcriptional repression by directly binding to the 3' untranslated region (UTR) of messenger RNAs (mRNAs), resulting in downregulation of their expression (Karabegović et al., 2017; Lu and Rothenberg, 2018). They play important roles in regulating different biological processes, including cell differentiation, proliferation, and apoptosis (Vidigal and Ventura, 2015). miRNA variants act as an oncogene or tumor suppressor gene indirectly (Ni et al., 2020). Single nucleotide polymorphisms (SNPs) of miRNAs may influence their functions through altering miRNA expression, maturation, and/or efficiency of targeting and, thereby, contribute to the risk of cancer (Zheng et al., 2017).

There is a controversy regarding the role of *miR-*

196a-2 in cancer. Some studies claimed that it has an oncogenic function. Others suggested that it acts as a tumor-suppressor. When it acts as an inhibitory factor of oncogenic molecules, it acts as a tumor suppressor and when it targets tumor suppressors, it acts as an oncogene (Chen et al., 2011).

miR-196a-2 polymorphism has significant associations with various types of cancer, including breast, lung, esophageal, gastric, and hepatocellular cancer (Alshatwi et al., 2012; Hu et al., 2008; Tutar, 2014; Peng et al., 2010 and Gawish et al., 2020). Carriers of the homozygote variant CC are more likely to develop gastric cancer compared with wild-type homozygote TT and heterozygote CT carriers and the C allele was significantly associated with lymph node metastasis of gastric cancer (Peng et al., 2010). Hu et al., (2008) reported significantly higher expression of *miR 196a* in non small cell lung tumor samples with CC genotypes compared with that of CT and TT individuals.

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Ovarian cancer is the fifth leading cause of cancer-related deaths among women worldwide (Siegel et al., 2019). Unfortunately, early detection tests are relatively lacking. Furthermore, most women with ovarian cancer are diagnosed in the late stages of the disease, which carries a poor prognosis (Xu et al., 2017 and Buchanan et al., 2017). Risk factors of ovarian cancer include early menarche, late menopause, low parity, lack of physical activity, higher body mass index, and long-term use of estrogen replacement therapy (Romero and Bast, 2012). Family history is an important risk factor which suggests that genetic factors contribute to the susceptibility to ovarian cancer (Norquist et al., 2015).

Early detection of ovarian cancer is difficult because its symptoms do not appear except in the late stages. Besides, screening modalities such as transvaginal ultrasound or serum cancer antigen 125 (CA125), are ineffective in early detection (Sun et al., 2017 and Lee et al., 2017). Despite the advancement of diagnostic techniques such as computed tomography/positron emission tomography scan and the use of targeted therapeutics, the 5-year survival rate ranges between ~30-50% (Suh et al., 2015).

So, seeking for new biomarkers for ovarian cancer detection and progress indication is important for the patients. We conducted the present study to evaluate the relationship between *miR-196a-2* gene polymorphism and ovarian cancer risk and prognosis in Egyptian females.

Materials and Methods

This study was conducted in the Departments of Obstetrics and Gynecology, Pathology and Medical Biochemistry & Molecular Biology - Faculty of Medicine, Zagazig University from December 2018 to December 2021. The study protocol was approved by the Institute Review Board of the Faculty of Medicine, Zagazig University. This is a case-control study. The participants were classified into 2 groups. Group A is the control group. It included 50 healthy females. Group B included 50 patients newly diagnosed with ovarian carcinoma confirmed by histopathological analysis. Informed consent was obtained from all participants.

All patients were subjected to the following: full history taking and complete physical examination. Routine laboratory investigations: complete blood count (CBC), liver and kidney function tests, and tumor marker CA 125 measurement were performed. Histopathological analysis for confirming ovarian carcinoma and immunohistochemistry for P53 were analyzed. Specimens of healthy ovaries were taken from cases with a total abdominal hysterectomy and bilateral salpingo-oophorectomy (TAH+BSO) received at the Pathology Department.

2 ml venous blood was taken on EDTA K2 containing tubes for DNA extraction. It was analyzed for the *miR-196a-2* polymorphism rs11614913. It was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) by restriction MspI. PCR was performed with a total volume of 25 µl with 100 ng DNA template, 2.5 µl of 10X PCR buffer, 1 U of Taq DNA polymerase, 0.2 mM dNTPs (Invitrogen, Carlsbad,

CA, USA), and 0.5 µmol/l of each primer (miR 196a 2 F 5' CCC CTT CCC TTC TCC TCC AGA TA 3' and R 5' CGAAAA CCG ACT GAT GTA ACT CCG 3'). The PCR conditions were 94°C for 5 min followed by 35 cycles of 30 sec at 94°C, 30 sec at 63°C, and 1 min at 72°C, and the final elongation step at 72°C for 10 min. A total of 10 µl PCR product was then digested using 2 µl (10 U/µl) MspI restriction enzyme (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) for 16 h at 37°C. The resulting fragments were separated by electrophoresis on a 3% agarose gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and visualized in three distinct patterns of restriction fragments. The CC genotype produced two fragments (125 and 24 bp), the TT homozygote produced one 149 bp fragment and the TC heterozygote produced three fragments (125, 149, and 24 bp). The experiment was performed in triplicate (Gawish et al., 2020).

Immunohistochemical staining was carried out using the polymer Envision detection system; the Dako EnVision™ kit (Dako, Copenhagen, Denmark). Tissue sections (3–5 µm) were deparaffinized in xylene and rehydrated in graded alcohol. To block endogenous peroxidase, slides were incubated for 10 min in hydrogen peroxide 3%. Dako target antigen retrieval solution (pH 6.0) was used. Then slides were incubated with Dako Mouse Primary Monoclonal (DO-7). The reaction was visualized by incubating the sections with diaminobenzidine (DAB) for 15 min then Mayer's hematoxylin was used. P53 nuclear stain in more than 5% of malignant cells was considered a positive immunoreactivity and its expression was evaluated as follows: p53-negative (≤ 5%), low p53 (5% to 50%), and high p53 (> 50%) (Lotfi et al., 2011).

Statistical analysis

Continuous variables were expressed as the mean ± SD and median (range), and the categorical variables were expressed as a number (percentage). Percentage of categorical variables were compared using Pearson's Chi-square test or Fisher's exact test when was appropriate. A P-value <0.05 was considered significant. All statistics were performed using SPSS version 22.0 for Windows (IBM Corp., Armonk, NY, USA) and MedCalc Statistical Software version 18.9.1 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2018).

Results

Clinicopathological features of patients with ovarian cancer

Low-grade serous ovarian carcinoma (LGSOC) represented 36% of cases while high-grade serous ovarian carcinoma (HGSOC) represented 64% of cases (Table 1). Regarding P53 expression, two patients (4%) showed focal expression, twenty patients (40%) showed negative expression, and twenty-eight (56%) patients showed diffuse expression (Table 1, Figures 1-3). The mean CA 125 level among ovarian cancer cases was 231.8 ± 251.8. There was a highly statistically significant increase of CA 125 levels among ovarian cancer cases than their controls (Table 1, Figure 4).

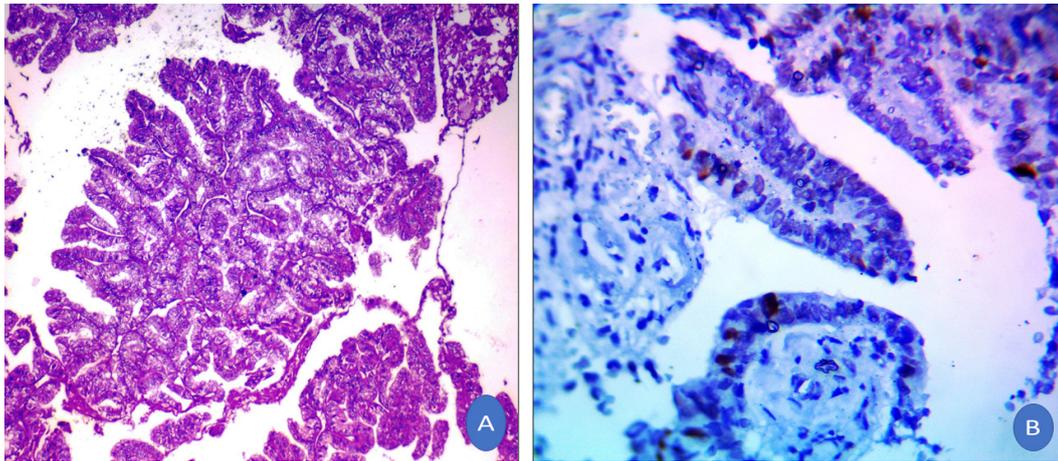


Figure 1. A, Histologic sections of low grade papillary serous carcinoma shows diffuse involvement by carcinoma with low to intermediate grade nuclei, prominent nucleoli, vesicular chromatin and moderate amounts of delicate cytoplasm and fibrovascular papillary core (H&E x200); B, Sections of low grade papillary serous carcinoma shows low P53 expression (IHC x400).

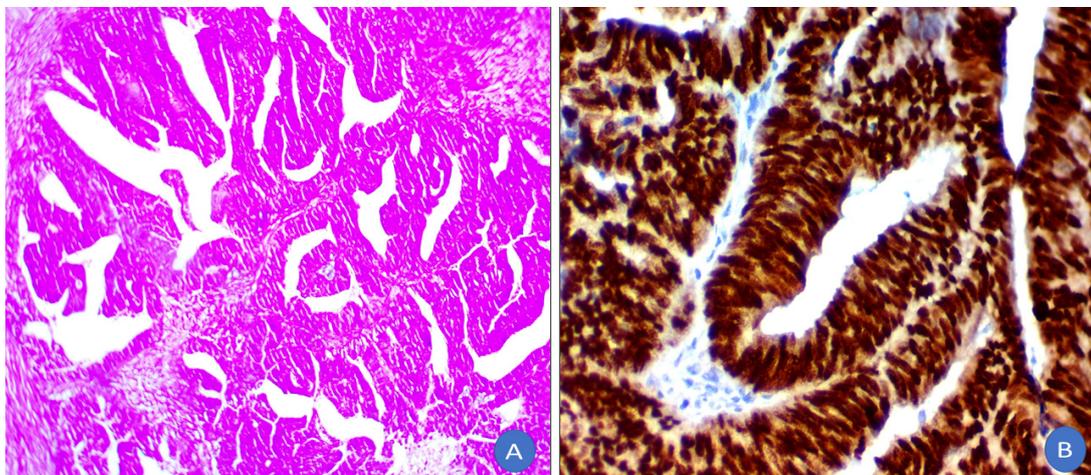


Figure 2. A, Histologic sections of intermediate grade papillary serous carcinoma shows diffuse involvement by carcinoma with intermediate grade nuclei, prominent nucleoli, hyperchromatic nuclei and moderate amounts of cytoplasm and fibrovascular papillary core (H&E x400); B, Sections of intermediate grade papillary serous carcinoma shows high P53 nuclear expression (IHC x400).

Table 1. Basic Characters of the Studied Population CBC, Liver and Kidney Function Tests

Variables	Mean \pm SD		t-test MW#	P-value
	Ovarian cancer N=50	Controls N=50		
Age (years)	47.3 \pm 10.7	42.9 \pm 9.45	2.19	0.03*
Hemoglobin (mg/dL)	10.5 \pm 0.89	11.7 \pm 1.13	5.86	<0.001**
RBCs (cells \times 10 ⁶ /uL)	4.12 \pm 0.49	4.64 \pm 0.46	5.42	<0.001**
WBCs (cells \times 10 ³ /uL)	7194.6 \pm 2331.3	7336.9 \pm 2735.1	0.14#	0.89
Platelets (cells \times 10 ³ /uL)	254.2 \pm 79.7	264.8 \pm 81.9	0.65	0.51
Random blood sugar (mg/dL)	124.8 \pm 29.7	108.5 \pm 43.3	4.76#	<0.001**
C Reactive protein	30.2 \pm 15.5	5.19 \pm 4.41	8.11#	<0.001**
Total bilirubin (mg/dL)	0.76 \pm 0.17	0.82 \pm 0.12	2.02	0.47
Direct bilirubin (mg/dL)	0.19 \pm 0.041	0.19 \pm 0.042	0.05	0.96
ALT (U/L)	32.7 \pm 8.49	28.2 \pm 7.24	2.85	0.005*
AST (U/L)	32.96 \pm 7.8	27.8 \pm 6.06	3.69	0.001*
Albumin (g/dL)	4.01 \pm 0.33	4.14 \pm 0.48	1.61	0.11
Creatinine (mg/dL)	1.1 \pm 0.19	0.99 \pm 0.19	1.8	0.08
Urea (mg/dL)	31.5 \pm 7.19	32.2 \pm 7.14	0.51	0.64

*, P-value<0.05 is significant; **, P-value<0.001 is highly significant

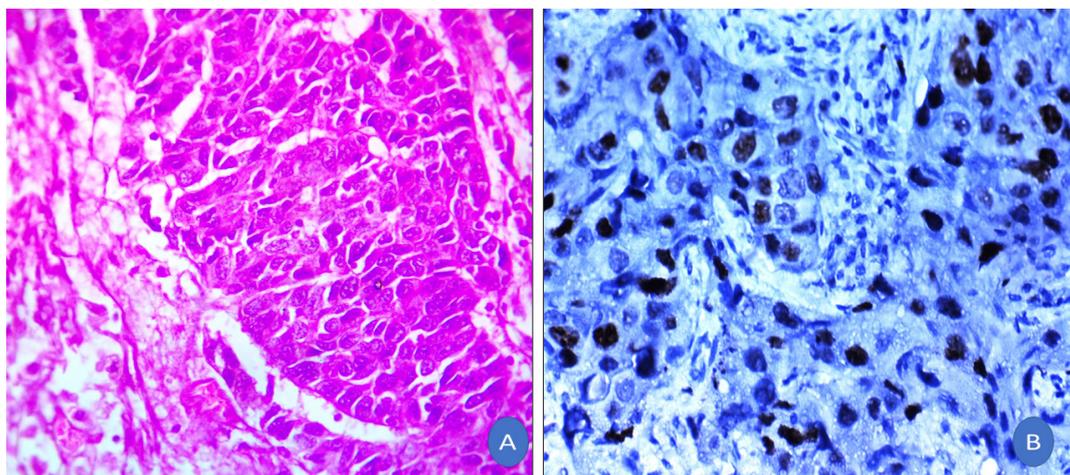


Figure 3. A, Histologic sections of high-grade ovarian carcinoma shows diffuse involvement by carcinoma with high grade nuclei, prominent nucleoli, hyperchromatic pleomorphic nuclei and moderate amounts of cytoplasm and fibrovascular papillary core (H&E x400); B, Sections of high-grade ovarian carcinoma shows high P53 nuclear expression (IHC x400).

Table 2. Difference in Tumor Marker CA 125 and Tumor Grading among Studied Ovarian Carcinoma Cases

Variables	Ovarian cancer N =50
CA 125	
Mean ± SD	231.8 ± 251.8
Median	55.5
(range)	(12 – 131)
Grade	N (%)
LGSOC	18 (36%)
HGSOC	32 (64%)
P -53	
Focal	2 (4%)
Complete absence	20 (40%)
Diffuse	28 (56%)
FIGO-3	
I	7 (14%)
II	17 (34%)
III	15 (30%)
IV	11 (22%)

miR 196 a 2 gene polymorphism in the two study groups

There was a statistically significant difference between ovarian cancer cases and controls regarding genotypes (P = 0.003). However, the distribution of the T and C alleles in both studied groups showed no significant

Table 3. Different Genotypes and Allele Distribution of miR-196 a-2 Variant among Both Studied Cases and Controls

Genotype	Group		X ²	P value
	Ovarian cancer (n=50)	Controls (n=50)		
CC	14	28	10	20
CT	26	52	14	48
TT	10	20	26	52
T allele	46	46	66	66
C allele	54	54	34	34

**P-value<0.001 (highly significant); * P-value<0.05 (significant)

difference (P = 0.17) (Table 2).

Relation between miR 196 a 2 gene polymorphism and clinicopathological features and There was a statistically significant increase in CA 125 levels among CT and CC genotypes carriers of ovarian cancer cases (p = 0.04) (Table 3). Besides, there was a statistically significant difference between miR-196 a-2 polymorphism and each of tumor grade, p53 immunohistochemical expression, and Figo classification (P < 0.001, 0.002, and < 0.001 respectively) (Table 4).

No statistically significant difference was found between miR-196 a-2 variants and any of the studied basic or laboratory characters of the ovarian cancer cases (Table 5).

Table 4. Relation between Tumor Marker CA-15, and Polymorphism of miR-196 a-2 Variant among Studied Ovarian Cancer Cases

Variables	Mean ± SD			KW	P-value
	TT N=10	CT N=26	CC N=14		
CA 15	42.6 ± 33.5	298.1 ± 306.3	243.6 ± 146.8	2.06	0.04*
Median	36.5	211.5	222		
(range)	4.8 – 127	18 - 1427	50 - 591		

*, P-value<0.05 (significant)

Table 5. Relation between Tumor Grading and Polymorphism of miR-196 a-2 Variant among Studied Ovarian Cancer Cases

Variables	Mean ± SD			KW#	P-value
	TT N=10	CT N=26	TT N=14		
Grade	N (%)	N (%)	N (%)	X ²	
LGSOC (n=18)	9 (50%)	8 (44.4%)	1 (5.6%)	18.1	<0.001**
HGSOC (n=32)	1 (3.1%)	18 (56.2%)	13 (40.6%)		
P53					
Focal (n=2)	2 (100%)	0	0 (0.0%)	16.7	0.002*
Complete ab. (n=20)	7 (35%)	10 (50%)	3 (15%)		
Diffuse (n=28)	1 (3.6%)	16 (57.1%)	11 (39.3%)		
FIGO 3					
I (n=7)	6 (85.7%)	1 (14.3%)	0 (0.0%)		
II (n=17)	4 (23.5%)	12 (70.6%)	1 (5.9%)	50.4	<0.001*
III (n=15)	0 (0.0%)	12 (80%)	3 (20%)		
IV (n=11)	0 (0.0%)	1 (9.1%)	10 (90.9%)		

*, P-value<0.05 is significant; **, P-value<0.001 is highly significant

Discussion

According to the cell origin, ovarian cancer is classified into epithelial, germ cell and stromal ovarian cancer. Other extremely rare cancers include small cell carcinoma and sarcomas (Boussios et al., 2017). Epithelial ovarian cancer (EOC) represents more than 85% of ovarian cancer cases and is the deadliest gynecological cancer, its major cause of death is mainly attributed to metastasis. EOC is further classified into five histological subtypes, including high-grade serous carcinomas (HGSC), low-grade serous carcinomas (LGSC), endometrioid carcinomas (EC), clear cell carcinomas (CCC), and mucinous carcinomas (MC).

In our study we focused on epithelial serous carcinomas.

In our study, there was a statistically significant difference between ovarian cancer cases and controls regarding genotypes. However, the distribution of T and C alleles in both studied groups showed no significant difference. Previous studies found no significant association between the *miR-196a-2* polymorphism and cancer risk. Lukács et al., (2019) found no significant difference between high-grade serous papillary ovarian cancer and controls regarding *miR-196a-2* genotypes or allele distribution. This result was similar to that found by Ni and Huang (2016) in ovarian cancer. Similar results were found by Chen et al., (2012) in colorectal cancer,

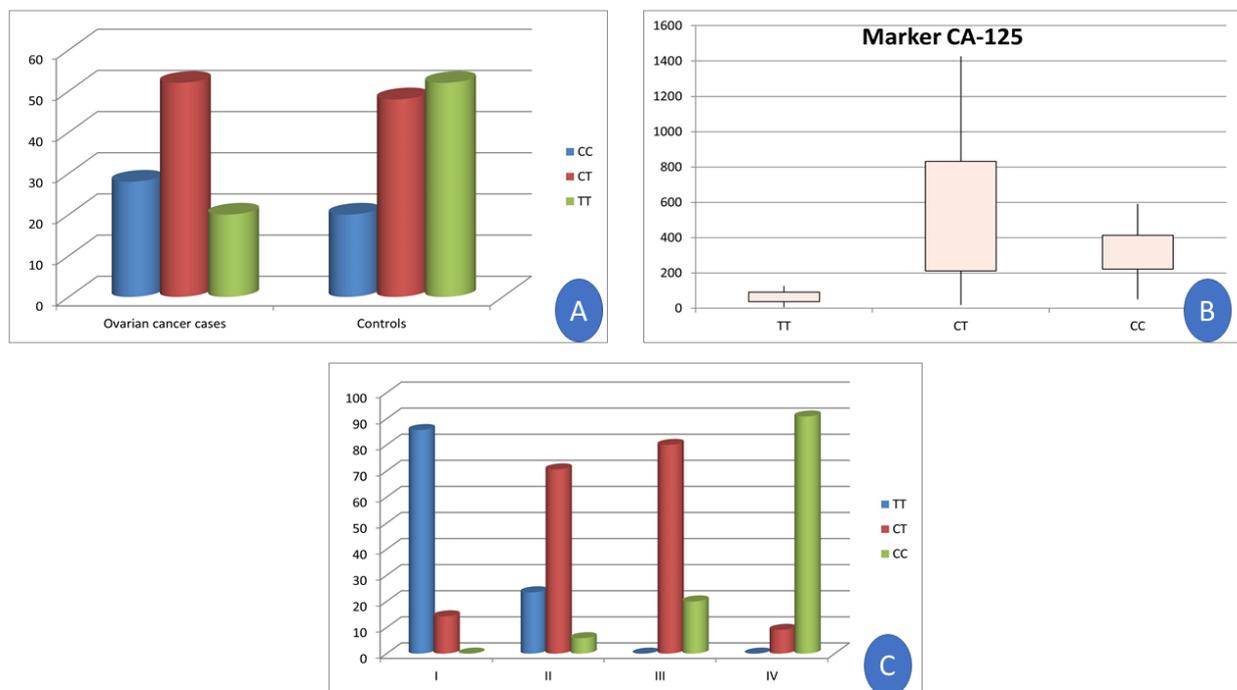


Figure 4. A, Genotypes distribution of miR-196 variant among both ovarian cancer cases and controls; B, Box-plot analysis of CA-125 among different genotypes of miR-196 a-2 variant of ovarian cancer cases; C, FIGO staging in relation to miR-196 variant genotypes among ovarian cancer cases.

Table 6. Relation between miR-196 a2 Variant Polymorphism and Basic Characters and Laboratory Tests of the Studied Cancer Cases

Variables	Mean \pm SD			t-test KW [#]	P-value
	TT N=10	CT N=26	CC N=14		
Age (years)	48.3 \pm 12.2 (30-66)	47.3 \pm 10.5 (33-69)	46.6 \pm 10.4 (30-64)	0.07	0.93
Hemoglobin (g/dL)	10.1 \pm 1.02 (8.9-12.3)	10.7 \pm 0.79 (8.8-12.1)	10.4 \pm 0.88 (9-11.8)	2.66	0.08
RBCs	4.17 \pm 0.56 (3.29-5.13)	4.16 \pm 0.53 (3.17-5.28)	4.02 \pm 0.38 (3.28-4.63)	0.42	0.66
WBCs	8055 \pm 2423.5 (4870-11970)	6899.2 \pm 2427.9 (1030-12150)	7128.6 \pm 2082.95 (4480-12680)	1.25 [#]	0.54
Platelets	237.2 \pm 63.4 (143-428)	256.4 \pm 89.8 (130-466)	262.9 \pm 73.2 (186-417)	0.35 [#]	0.84
RBS	133.6 \pm 51.1 (98-275)	122.5 \pm 23.5 (87-211)	123 \pm 19.6 (94-172)	0.27 [#]	0.11
CRP	34.7 \pm 17.2 11-64	30.9 \pm 14.4 6-75	25.7 \pm 16.2 9-68	3.13 [#]	0.21
Total bilirubin (mg/dL)	0.8 \pm 0.08 (0.68-0.95)	0.76 \pm 0.17 (0.37-1.1)	0.73 \pm 0.21 (0.49-1.27)	0.45	0.74
Direct bilirubin (mg/dL)	0.19 \pm 0.03 (0.14-0.23)	0.19 \pm 0.05 (0.12-0.27)	0.19 \pm 0.04 (0.13-0.31)	0.05	0.96
ALT (U/L)	31.7 \pm 6.49 (21-45)	31.2 \pm 8.64 (17-48)	35.8 \pm 8.82 (18-47)	1.41	0.25
AST (U/L)	29.6 \pm 6.71 (19-41)	33.2 \pm 7.98 (16-51)	34.9 \pm 7.97 (19-45)	1.29	0.29
Albumin (g/dL)	3.98 \pm 0.21 (3.6-4.2)	4.01 \pm 0.36 (3.2-4.6)	4 \pm 0.38 (3.5-4.6)	0.03	0.97
Creatinine (mg/dL)	1.16 \pm 0.21 (0.79-1.46)	1.01 \pm 0.19 (0.74-1.5)	1.1 \pm 0.15 (0.71-1.25)	2.42	0.11
Urea (mg/dL)	34.9 \pm 7.61 (28-53)	30.3 \pm 6.99 (18-48)	31.2 \pm 6.97 (19-41)	1.51	0.24

P-value>0.05 (not significant)

Deng et al., (2015) in bladder cancer, and Pu et al., (2014) in gastric cancer. On the other hand, Song et al., (2016) observed that the CC genotype increased ovarian cancer risk compared with those carrying the wild-type TT and heterozygous CT genotypes. Moreover, they found increased production of mature miR 196a-2 in the C allele carriers compared to the T allele carriers and they considered that responsible for the abnormal cell viability and migration/invasion capacity in the human ovarian cell line. They explained that rs11614913 polymorphism may affect the processing of the pre miRNA to its mature form. Also, Liu et al., (2015) found that miR-196a2 polymorphism can influence the susceptibility to ovarian cancer in a Chinese population.

In our study, there was a statistically significant difference between miR-196 a-2 polymorphism and each of tumor grade, p53 immunohistochemical expression, and Figo classification which indicated the association of miR-196 a-2 polymorphism with poor prognosis in ovarian cancer. Fan et al., (2015) reported the association

between high levels of miR-196a expression and worse overall survival in ovarian cancer patients, especially in advanced-stage tumors. miR-196a expression was positively correlated with tumor stage and lymph node metastasis.

miR-196a-2 role in carcinogenesis is by targeting many genes, such as lamin B receptor (LBR), rab4 interacting protein (RUFY2), autophagy-related 9a (ATG9A), methyl CpG binding domain 4 (MBD4), HOX gene, HMGA2, and annexin A1 (Rapado-González et al., 2019 and Lukács et al., 2019). Also, Ni et al (2020) found that miRNA-196a promotes cell proliferation and inhibits apoptosis in human ovarian cancer by directly targeting DDX3 and regulating the PTEN/ PI3K/AKT signaling pathway.

In conclusion, There was a statistically significant increase of CA 125 levels among C allele carriers of ovarian cancer cases. Besides, there was a statistically significant association between the *miR-196a-2* polymorphism and each of tumor grade, p53 immunohistochemical expression, and Figo classification. So, *miR-196a-2*

polymorphism can be a possible prognostic factor in ovarian cancer.

Author Contribution Statement

Conception: Ahmed Algazeery and Samia Hussein; Interpretation or analysis of data: Ahmed Algazeery, Reham Sameh, Amira S. Al-Karamany, and Samia Hussein; Preparation of the manuscript: Samia Hussein; Revision for important intellectual content: All authors; Supervision: Ahmed Algazeery and Samia Hussein.

Acknowledgements

Ethical Approval

The experimental protocol was approved by the Faculty of Science, Zagazig University, Zagazig, Egypt.

Availability of data

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interests

the authors declare no conflict of interest.

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