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Vascular endothelial growth factor gene expression and polymorphism C936T in Iraqi patients with ovarian carcinoma

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Abstract: The present study aimed to shed light on the association between the angiogenic factor VEGF gene expression and its genetic polymorphism *VEGF* C936T in the angiogenesis of ovarian cancer. A total of 44 Paraffin-embedded tissue blocks from patients with different stages of newly diagnosed ovarian cancer were provided by certain Iraqi hospitals as well as 14 samples of patients with benign ovarian tumors tissues as a control group were used in this study. The results detected that *VEGF* mRNA in ovarian cancer samples was statistically significant compared to benign tumors(p value=0.039<0.05). The samples were divided into high and low mRNA-expression depending on the mean value of *VEGF* gene expression in benign tumors which used as a cutoff, the results showed that high statistical significant differences (P value = 0.0023<0.01) between high mRNA-expressing samples 30(76.9%), and low mRNA-expressing samples 9(23.07%). Statistical no significant differences were detected in compare with other histopathology tumor types. In correlation with stages, statistically significant difference was found between 31 (79.4%) of samples with stage I which showed the highest level of expression(P=0.0210<0.05). The result of *VEGF* C936T polymorphism showed that out of 42 ovarian cancer patients 24(57.14%) were homozygous for the C/C genotype, 11(26.2%) were heterozygous C/T and 7(16.66%) were homozygous T/T. High significant prevalence of the *VEGF* 936C allele was detected in both ovarian cancer patients (P value 0.0019 <0.01) and benign ovarian tumors (P value 0.0026 <0.01) as compared with *VEGF* 936T allele. The study showed that the average of *VEGF* gene expression in ovarian cancer patients carrying the +936CT+TT genotypes (1.815116, 2.298769) was significantly lower than that in ovarian cancer patients with the *VEGF*+936CC genotype (4.395884). Same results were obtained from patients with benign ovarian tumors. In conclusion the present study investigated that the presence of the *VEGF*+936 T allele is associated with a decreased risk of ovarian cancer since the C936T polymorphism has been reported to be associated with lower *VEGF* plasma levels.

Key words: Ovarian cancer, Angiogenesis, VEGF, *VEGF* C936T.

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Introduction:

Vascular development in the ovary is a cyclic process, spatially and temporally defined. A vascular primordial follicles depend on proximity to stromal vessels for nutrients. During the transition from an a vascular, primary follicle to a vascular secondary follicle, angiogenesis occurs in the theca layer. How the secondary follicle becomes endowed with vasculature is unclear. This transition may be due to local transformation of

mesenchymal cells into endothelial cells or active migration of endothelial cell precursors from pre existing blood vessels. *VEGF* and members of the *VEGF* gene family play a fundamental role in growth and differentiation of vascular and lymphatic endothelial cells (1). In the human ovary, the *VEGF* receptor is primarily detected in the theca interna of antral and developing follicles and the intensity of *VEGF* expression is higher in more mature follicles (2). *VEGF* activity has not been detected in primordial, primary or atretic follicles. Inhibition of *VEGF* and *VEGF* receptor activity has helped to elucidate the role of this angiogenic molecule during folliculogenesis and corpus luteum formation and function. The different species of mRNA that encode for the *VEGF* protein have been identified and are found to be expressed in a tissue-specific manner. These specific mRNA species increase from differential splicing, with the 165 amino acid form of *VEGF* lacking sequences encoded by exon 6, and the 121 amino acid form lacking exon 6 and 7 sequences (3). Ovarian tumors, like many malignancies, over express proangiogenic factors including vascular endothelial growth factors (*VEGF*s), fibroblast growth factors, angiopoietin, platelet-derived growth factors (PDGFs), and pro-angiogenic cytokines such as tumor necrosis factor alpha and interleukins 6 (4). Of these, members of the *VEGF* family are the most potent pro-angiogenic factors. *VEGF* activation promotes endothelial cell proliferation and migration for the formation of new blood vessels and increases permeability of existing blood vessels to allow for the leakage of multiple plasma proteins, including those playing a role in angiogenesis (5). Also, *VEGF* inhibits apoptosis of the newly formed hyperpermeable blood vessels. By this mechanism, *VEGF* also plays a key role in the formation of ascites and pleural effusions. Additionally, *VEGF* plays a significant part in the normal function of the ovary, with serum *VEGF* levels rising and falling in a predictable pattern during the ovulatory cycle (6). Therefore, it is not surprising that *VEGF* plays a role in the biology of ovarian cancer. On the other hand many studies have investigated the role of *VEGF* polymorphisms as genetic determinant for susceptibility and outcome of breast, prostate, NSCL and colorectal cancer. Several polymorphisms have been reported within the promoter (-2578C>A, −2489C>T, −1498C>T and −1154G>A), 5’-UTR (-634G>C and −7C>T) and 3’-UTR (936C>T and 1612G>A) for the *VEGF* gene. The variant allele for −1154G>A and 936C>T results in lower VEGF expression, whereas the variant allele for −1498C>T and −7C>T results in increased concentrations of VEGF mRNA. The functional role of −2578C>A and −634G>C is not in agreement among studies, which report low or high VEGF production for variant alleles (7). In the present study we aimed to shed light on the association between the angiogenic factor VEGF gene expression and its genetic polymorphism *VEGF* C936T in the angiogenesis of ovarian cancer.

Materials and Methods:

Subjects and samples collection:

The tissue samples used in this study included 44 Paraffin-embedded tissue blocks from patients with

different stages of newly diagnosed Invasive ovarian cancer were provided by certain Iraqi hospitals (including Al-Kadhemia , AL -Yarmouk Teaching Hospital, Baghdad Hospital, the Teaching Laboratories of Medical City, Nuclear Medical Hospital in Baghdad and Alsader Hospital in Missan) after patients underwent to total abdominal hysterectomy and bilateral salpangio-oopherectomy(TAH-BSO), subtotal abdominal hysterectomy, vaginal hysterectomy, and endometrial biopsy, 14 samples of patients with benign ovarian tumors tissues were used as a control. The required information about the patients and the histopathologic properties of the tumors were recorded from the patients' files. The Paraffin-embedded tissue blocks were sectioned into 10µm in DNase-RNase tubes for molecular evaluation. Samples subjected to RNA extraction and molecular study by using Revers Transcription and Real Time PCR at Molecular Oncology Unit in Guy´s hospital – Kings college/London.

RNA extraction, reverse transcription:

Total RNA was extracted from benign and malignant tumor tissues and normal tumors was extracted using the RNeasy FFPE Kit which designed for purifying total RNA from FFPE tissue sections (Qiagen-*USA*) according to the protocol provided by the manufacturer. Total RNA was reversely transcribed using Thermo-Script™ Reverse Transcription kit (Invitrogen/USA). The procedure was carried out in a reaction volume of 50 µl composed of 15 µl Denaturized RNA,0.2 µl Random hexamere primers 3µg/µl, 5µl of 10 mM dNTP Mix, 10µl of 5x cDNA synthesis buffer, 2.5µl RNase OUT (40U/µl), 2.5µl ThermoScript RT (15 units/µl), 14.8µl DEPC-treated water. The samples were then placed in a 96 Well Thermal Cycler, and cycled at the following conditions: 25ºC for 10 min., 10 min. at 37ºC ,60 min. at 42ºC followed by 75ºC for 5 minutes. The converted cDNA. was stored at -80°C and used as a template for PCR amplification of VEGF. Primers and probes were designed using Primer Express software (ABI, USA). The primers and prop for *VEGF* were as follows:5'AACACAGACTCGCGTTG CAA3'(foreword),5'CGGCTTGTCACA TCTGCAAGT3'(reverse),5'CGAGGCA GCTTGAGTTAAACGAACG-3(prop). PGK1 gene used as an endogenous control gene. The amplification of VEGFC cDNA for real time RCR analysis was performed in duplicate using the Applied Bio systems 7900. The 25 µl of reaction volume containing 10 µl of master mix ,3 µl of primer mixes, 3µl of RNase free water and 4µl of cDNA template. Real-Time PCR protocol was as follows; stage 1 50ºC for 1 minutes, (stage 2: 95ºC for 45 sec., 55C for 45 Sec. and 72ºC for 1 min.) repeated for 32 cycles. The slope of a standard curves was used to estimate the PCR amplification efficiency of a real-time PCR reaction. A calculation for estimating the efficiency (E) of a real-time PCR assay was performed as following:

$E = (10^{-1}/\text{slope} - 1) \times 100$

$E = (10^{-1}/3.35 - 1) \times 100$

For each sample, the cycle threshold (Ct) is defined as the number of PCR cycles required to achieve the user defined level of fluorescence. This Ct value is used to compare across all samples. The Ct is inversely proportional to the amount of starting

mRNA of the target gene (*VEGF*) as well as the endogenous control gene (*PGK1*). The relative fold change ratio of the target gene in the sample was calculated as described below:

$\text{Log copy}_{(\text{enodogenus control gene})} = (\text{Ct} - 32.85) / -3.3592$

$\text{Copy number}_{(\text{enodogenus control gene})} = 10^{\wedge}\text{Log copy}$

$\text{Log copy}_{(VEGF)} = (\text{Ct} - 34.82) / -3.5126$

$\text{Copy number}_{(VEGF)} = 10^{\wedge}\text{Log copy}$

$\text{Fold change} = \text{Copy number}_{(VEGF)} / \text{Copy number}_{(\text{enodogenus control gene})}$

DNA extraction, Genotyping and Sequencing analysis:

DNA was extracted and purified from Formalin-Fixed Paraffin-Embedded (FFPE) tissue sections of 58 ovarian tumor samples with QIAamp DNA FFPE Tissue Mini Kit (Qiagen,USA) which was designed for purifying of DNA from FFPE tissue sections. The primers for *VEGF* -+936C/T were as follows: 5'-GAAATGAAGGAAGAGGAGACTCTG-3' (forward) and 5'-TCCCAGAAATAAAACTCTCTAATCTTC-3' (reverse). The procedure was carried out in a reaction volume of 20 µl. The PCRs conditions were as follows: an initial step of 10 min at 95°C foe enzyme activation, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s for denaturation, annealing and extension respectively, followed by final extension for 5 min. at 72°C, and then incubation at 4°C to time end. The resulting DNA fragments were separated by 2% agarose gel electrophoresis and visualized under UV light after ethidium staining. Before sequencing the PCR products purified by using Charge Switch PCR Clean-up kit according to the manufacturer's instructions, The sequencing performed using ABI BigDye terminator ready reactions Kit (Applied Biosystems, USA) and ABI Automated DNA Sequencer 3730. Data were analyzed using Mutation Surveyor Software of sequencing reading Version 3.24.

Statistical Analysis:

The Statistical Analysis System-SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage and Least significant difference –LSD test was used to significant compare between means in this study.

Results:

A total of 58 samples of 44 samples with ovarian cancer and 14 benign ovarian tumors tissues were examined for the expression of *VEGF* art-PCR. The patients' age range was 14-70 years and the median is 47 years. According to the family history, all samples were negative for family history to the ovarian cancer. Clinical features of ovarian cancer samples are listed in table 1. In regard to the menopausal state of ovarian cancer patients, 20(46.5%) of samples were premenopausal, while 23(53.48%) of them were postmenopausal. According to the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system, most of samples 35(81.4%) came with stage I, while the other 8 (18.6%) samples were with stage III. According to the tumor histological types, the samples were divided into three clinical groups; surface epithelial tumors 38(88.3%) samples, sex cord tumors 3(6.9%)

samples, and germ cell tumors 2(4.65%) samples. qRT-PCR analysis revealed that *VEGF* mRNA was expressed in the tissues of both malignant and benign tumors (figure 1). The present study showed that *VEGF* mRNA was expressed in 50 (86.2%) of samples, 39 samples with ovarian cancer and 11 benign ovarian. The higher level *VEGF* mRNA in ovarian cancer samples was statistically significant (Mean ± SE: 4.01 ± 0.80, p value=0.039<0.05) compared to benign tumors (Mean ± SE: 0.906 ± 0.17). The mean value of *VEGF* gene expression in benign tumors was used as a cutoff to separate tumors into high and low mRNA-expressing samples which showed high statistically significant differences (P value = 0.0023<0.01) between high mRNA-expressing 30(76.9%), and low mRNA-expressing samples 9(23.07%). Although the Endometriod ovarian tumors showed the highest level of *VEGF* gene expression (Mean ± SE: 6.90 ± 3.68), statistically no significant differences were detected in compare with other histopathologic tumor types. In correlation with stages, statistically significant difference was found between 31(79.4%) of samples with stage I which showed the highest level of expression (Mean ± SE: 4.47 ± 1.12, P=0.0210<0.05) and 8 (20.5%) samples with stage III (Mean ± SE: 1.66 ± 0.65).

The results of genotyping in benign and ovarian cancer patients viewed in (table 2). The result of *VEGF* C936T polymorphism showed that out of 42 ovarian cancer patients 24(57.14%) were homozygous for the C/C genotype, 11(26.2%) were homozygous T/T and 7(16.66%) were heterozygous C/T (figure 2). For patients with benign ovarian tumors, 6(50%) were homozygous for the C/C genotype, 3(25%) were heterozygous C/T and 3(25%) were homozygous T/T. High significant prevalence of the *VEGF* 936C allele was detected in both ovarian cancer patients (P value 0.0019 <0.01) and patients with benign ovarian tumors (P value 0.0026 <0.01) as compared with *VEGF* 936T allele. In compare with *VEGF* gene expression, the study showed that the average of gene expression in ovarian cancer patients carrying the +936CT+TT genotypes (1.815116, 2.298769) was significantly lower than that in ovarian cancer patients with the *VEGF*+936CC genotype (4.395884). Same results were obtained from patients with benign ovarian tumors. The distribution of the three *VEGF* C936T genotypes was analyzed in correlation with stage of cancer. The results showed that for patients with stage I the percentages of these genotypes were 60%, 28%, and12% for the genotypes CC, TT, and CT respectively, while for patients with stage III the percentages of these genotypes were 50%, 33%, and17% for these genotype. The CC genotypes in patients with stage I showed highly statistically significant among other genotypes (P value 0.0017 < 0.01).

Discussion:

Among the pro-angiogenic factors, *VEGF* is recognized as the predominant mediator of angiogenesis in tumor cells (8). As *VEGF* is overexpressed in most ovarian cancers, the *VEGF* pathway is a promising target for anti-angiogenic therapy against ovarian cancer (9). The present study aimed to investigate the expression and genetic variations of VEGF gene and its relevance to the risk for ovarian cancer. A single-nucleotide polymorphisms (SNPs) was selected

(*VEGF*-936), the selection of this polymorphisms was based on its sufficient frequency in the general population, as well as previous evidence indicating its clinical relevance.

The present study showed that *VEGF* mRNA was expressed in 50 (86.2%) of samples, 39 samples with ovarian cancer and 11 benign ovarian. The higher level *VEGF* mRNA in ovarian cancer samples was statistically significant (p value=0.039<0.05) compared to benign tumors. These findings are consistent with the data reported by Shen *et al*., (10) who showed that high VEGF expression was significantly associated with ovarian carcinomas as compared to benign or borderline ovarian tumors. Wang *et al*., (11) showed that in contrast to benign and borderline tumors, VEGF was detected in 80% of ovarian carcinomas, with most tumor cells showing strong and diffuse expression. He also reported that the increment of VEGF expression from benign to malignant ovarian epithelial tumors was statistically significant. Smerdel *et al*., (12) reported that *VEGF* gene expression differed among normal ovaries, benign, and malignant tumors with significantly higher levels of *VEGF* mRNA in malignant tumors. The results were inconsistent with those reported by Zhang *et al*.,(13) who showed that no significant difference was identified in *VEGF* mRNA among benign and low malignant potential neoplasms, as well as early stage (stage I) of ovarian cancer.

Although the Endometriod ovarian tumors showed the highest level of *VEGF* gene expression (Mean ± SE: 6.90 ± 3.68), statistically no significant differences were detected in compare with other histopathologic tumor types. These results were similar to those reported by Wang *et al*.,(11) who did not find any statistically significant difference among histological types of ovarian tumor in *VEGF* expression. Shen *et al*.,(10) showed that no significant difference was found between the negative, low and high *VEGF* expressions and tumor histological types patient age, and tumor size. While the present study results are different from that obtained by Chen *et al*.,(14) who showed that mRNA expression level of *VEGF* gene was significantly higher in ovarian serous cancer than in other ovarian epithelial cancers.

In correlation with stages, statistically significant difference was found between 31 (79.4%) of samples with stage I which showed the and 8 (20.5%) samples with stage III. Most of studies related with *VEGF* gene expression indicated that *VEGF* mRNA either positively correlated with clinic pathological staging(10,14,15), or showed that no significant difference in *VEGF* expression with cancer stages. Wang *et al*.,(11) reported that there was no any statistically significant difference in the percentage of *VEGF* expression in both epithelial and stromal compartments among different tumor stages in malignant ovarian epithelial tumors. This study is one of the very few studies that showed down regulation of *VEGF* mRNA in late cancer stage compared with early stage, as Zhang *et al*.,(13) study that showed the *VEGF* mRNA levels further decreased in late stage compared with early stage of ovarian carcinomas. Quentin *et al*.,(16) who performed his study on alteration of the *VEGF* in transitional cell carcinomas (TCC) of the urinary bladder, showed that

significant relationship was found between tumor stages and expression , low-stage superficial TCC revealing an approximately 2-fold elevated expression compared to high-stage. The mechanisms underlying down-regulation of *VEGF* gene activity in high cancer stage are not clear. Many hypotheses have been suggested to attribute this *VEGF* mRNA down regulation in late cancer stages, including that *VEGF* likely plays a marginal role in promoting angiogenesis in advanced stage disease. Ovarian carcinomas may exhibit an abundant rich stroma component, which may be highly cellular. It has been postulated that stroma-epithelial cell interactions may inhibit the growth of tumorigenic surface epithelium in early stages of ovarian oncogenesis (17). It is possible that the overall influence of ovarian steroidogenic stroma on tumor development might be inhibitory, resulting in its progressive loss during malignant progression, which might provide an explanation for the decline of *VEGF* expression (13). The decreased expression of *VEGF* mRNA in advanced high-stage may point to a reduced vascular destabilization and a concomitant diminished recruitment of new blood vessels, suggesting a balance between vessel regression and vascular growth with a less pronounced vascular remodeling during late stages of carcinogenesis(16).

Because the contribution of *VEGF* 936C>T polymorphism to oncogenesis has been investigated in several types of cancer, the relationship between the *VEGF* 936C>T polymorphism and ovarian cancer patient were evaluated. The result of *VEGF* C936T polymorphism showed that 24 out of 42 ovarian cancer patients (57.14%) were homozygous for the C/C genotype, 11 patients (26.2%) were homozygous T/T and 7 patients(16.66%) were heterozygous C/T. For patients with benign ovarian tumors six patients (50%) were homozygous for the C/C genotype , 3 patients (25%)were heterozygous C/T and 3 patients(25%) were homozygous T/T. The genotype frequencies of these three polymorphisms in the current study were different from those reported by Schultheis *et al*.,(18) who found that 69.8% of patients were homozygous for the C/C genotype, 26,4%were heterozygous C/T and 3.77% patients were homozygous T/T. Other study performed by Magdoud *et al*.,(19) on the patients with recurrent spontaneous miscarriages showed that the frequencies of these three polymorphisms were 68.1%, 28.6% and 3.3%. The study performed by Kim *et al*.,(20) on the patients with colorectal cancer showed that the frequencies of these three polymorphisms were 62.%, 34.2% and 3.8% , the study performed by Lurje *et al*.,(21) on the patients with stage III of colon cancer showed that the frequencies of these three polymorphisms were 66.1% , 30.6% and 3.3% and the study performed by Hsiao *et al*.,(22) on the patients with thyroid cancer which showed that the frequencies of these three polymorphisms were 59.4.%, 36.5% and 4.2% for the 936CC +936CT and TT genotypes respectively. The present study results were similar to the previous studies by showing that the polymorphism 936CC was the most frequent genotype (57.14%), while it's differs from other studies in the frequent of the other two genotypes. These differences attributed either to the size of samples used in present study was

less than those used in previous studies or its related with environmental factors and geographical disruption of the samples which showed that the frequent of these genotypes in Iraqi patients it differs from that obtained from the previous studies which performed in USA, Tunisia, and West Asia respectively.

High significant prevalence of the *VEGF* 936C allele was detected in both ovarian cancer patients (P value 0.0019 <0.01) and patients with benign ovarian tumors (P value 0.0026 <0.01) as compared with *VEGF* 936T allele. These results were similar with those reported by Jiang *et al*.,(23) who found that the C allele frequency was higher in both control an AMD patients (82.8%,78.5%) than the frequency of T allele (17.3%,21.5%), Ke *et al*.,(24) results also showed that the frequencies of T allele were significantly lower than C allele in both control and gastric cancer patients (16.6%,16.9%). Hsiao *et al*.,(22) reported a significantly lower frequency of the 936T allele in both control and patients with thyroid cancer (20.9%,22.4%) than that of 936C allele (79.1%,77.6%).

The association between the *VEGF*+936 genotype and level of *VEGF* gene expression was examined, since this SNP has been proven to correlate with the under-expression of the gene, significantly lowering the plasma VEGF levels in carriers of at least one *VEGF* 936T allele. The study showed that the average of *VEGF* gene expression in ovarian cancer patients carrying the *VEGF* +936CT+TT genotypes (1.815116, 2.298769) was significantly lower than that in ovarian cancer patients with the *VEGF*+936CC genotype (4.395884) .The same results were obtained from patients with benign ovarian tumors average of *VEGF* gene expression in individuals carrying the +936CT+TT genotypes was significantly lower (1.715849, 0.570662) than that in ovarian cancer patients with the *VEGF*+936CC genotype (3.292324).

The results showed that the +936CT/TT genotypes were significantly correlated with lower levels of *VEGF* gene expression in both groups of patients with malignant and benign ovarian tumors. These results were similar to that obtained by Prabha *et al*.,(25) who showed that placental *VEGFA* +936 CT+TT was associated with a 36% reduction in first-trimester placental *VEGF*A mRNA levels compared with the CC genotype. Krippl *et al*.,(26) found that plasma levels of *VEGF* were significantly lower in 936T allele carriers than in noncarriers. Our results is differed from that reported by Faffe *et al*.,(27) who showed that the +936 C/T genotype had no impact on *VEGF* release. The present study investigated that the presence of the *VEGF*+936 T allele is associated with a decreased risk of ovarian cancer since the C936T polymorphism has been reported to be associated with lower *VEGF* plasma levels. Those who are homozygous for TT have lower *VEGF* production compared with the CC genotype, which, in turn, may decrease the risk of tumor development.

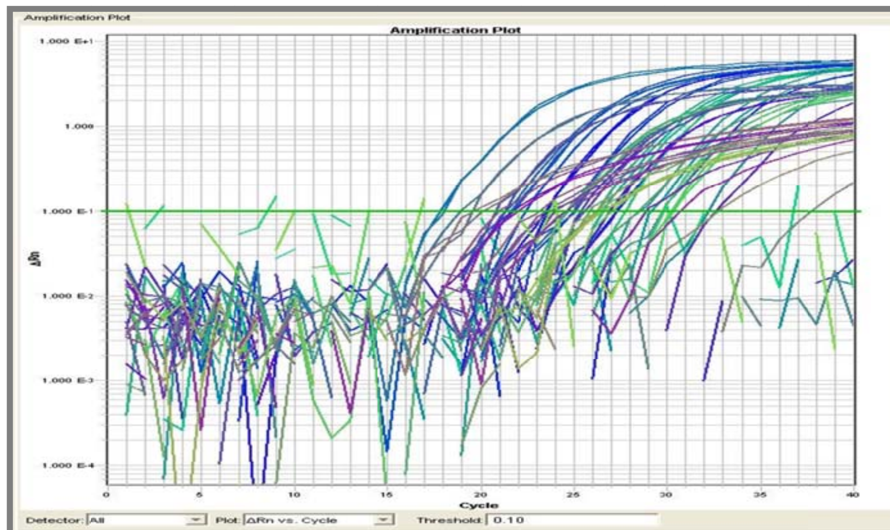

Figure (1): Amplification target and endogenous gene product run with duplicate samples Cycle number is plotted on the x-axis with level of fluorescence on the y-axis. The threshold fluorescence level is depicted by the green line.

Table (1): Clinical features of ovarian cancer samples.

Age groups	
children age 0-14 years	2(4.65%)
Teenagers and young adults aged 15-24 years	1(2.32%)
Adults aged 25-49 years	16(37.2%)
Adults aged 50-74 years	24(55.8%)
Menopausal state	
premenopausal no. (%)	20(46.5%)
postmenopausal no. (%)	23(53.48%)
Family history	
Positive no. (%)	0
Negative no. (%)	43(100%)
FIGO surgical stage	
Stage I no. (%)	35(81.4%)
Stage III no. (%)	8 (18.6%)
Tumor histological types	
Surface epithelial tumors	38(88.37%)
Sex cord tumors	3(6.9%)
Germ cell tumors	2(4.65%)
Age groups	
children age 0-14 years	2(4.65%)
Teenagers and young adults aged 15-24 years	1(2.32%)
Adults aged 25-49 years	16(37.2%)
Adults aged 50-74 years	24(55.8%)

Menopausal state	
premenopausal no. (%)	20(46.5%)
postmenopausal no. (%)	23(53.48%)
Family history	
Positive no. (%)	0
Negative no. (%)	43(100%)
FIGO surgical stage	
Stage I no. (%)	35(81.4%)
Stage III no. (%)	8 (18.6%)
Tumor histological types	
Surface epithelial tumors	38(88.37%)
Sex cord tumors	3(6.9%)
Germ cell tumors	2(4.65%)

Table (2): The results of VEGF C936T polymorphisms in benign ovarian tumors and ovarian cancer patients.

***VEGF* C936T**	**GENOTYPES**			**Alleles**		**P Value**
	CT	**CC**	**TT**	**T**	**C**	
Benign tumors NO.	**3**	**6**	**3**	**37.5%**	**62.5%**	**0.0026 ****
%	**25%**	**50%**	**25%**			
Ovarian cancer NO.	**7**	**24**	**11**	**34.53%**	**65.47%**	**0.0019 ****
%	**16.66%**	**57.14%**	**26.2%**			
P <0.01						

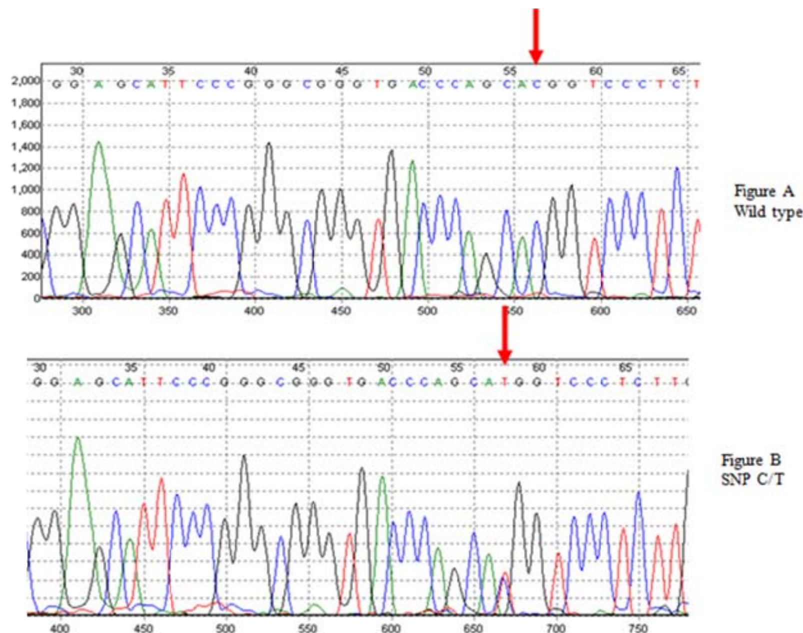

Figure (2): Example of DNA sequencing results showing the wild type of *VEGF*+9C936T polymorphisms and the transition of the wild type allel C in figure a to T in figure b. The arrow refer to the change in single nucleotide in the sequence:

5-AGCATTCCCGGGCGGGTGACCCAGCAC/TGGTCCCTCTTGGAATTGGATTCGCC-3

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