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Evaluation of *p53* and *K-ras* Gene Mutations Frequency in Iraqi Women with Ovarian Carcinoma

Maisaa Gazi Jumaa

College of Medicine, Maisan University, Maisan, Iraq

Abstract: Ovarian cancer represents the fourth most frequent type of cancer among females and is the leading cause of death from gynecological cancer in the western world. More recently, ovarian tumors have been broadly classified into two distinct groups with unique histological, clinical and molecular profiles. Type I tumors in which *BRAF* and *K-ras* somatic mutations are relatively common, and type II tumors which display high levels of genomic instability with few common mutations, other than *TP53*, which is altered in over 90% of the cases. In the present study 58 samples with newly diagnosed ovarian cancer were analyzed for detecting the frequency of *p53* and *K-ras* gene mutations in Iraqi ovarian cancer patients, as well as 15 samples of apparently healthy women used as a control group. The analysis was based on conventional PCR amplification of exons 5 and 7 of the *p53* gene and codon 12 of *K-ras*. For both p53 and *K-ras* genes, none of healthy control exhibited mutation in those genes. *p53* mutations detected in 13(22.4%) of ovarian cancer samples, which was significantly higher in compare with healthy controls ($p<0.05$). The results showed that out of thirteen mutant ovarian cancer samples, exon-5 mutation was the most frequent and detected in 10 (76.9 %), followed by exon-7 that detected only in 3(23.07%) of cases. Statistically there were no significant differences in mutational rates of *p53* gene in patients with age, menopausal state, tumor histological subtypes, and different FIGO stages. *K-ras* mutation detected in only 3(5.17%) of ovarian cancer samples. There were no significant difference in mutational rates of *K-ras* gene in patients with age, menopausal state, tumor histological subtypes, and different FIGO stages, but all these three mutant samples with stage I. Out of 58 samples only one patient 1(1.7%) have been identified with mutations in both genes. In conclusion, the present study results show that mutations of the *p53* gene are not rare events, and *K-ras* mutations status is not a prognostic factor in ovarian carcinomas.

Key words: Ovarian tumor, *p53*, *K-ras*, mutations.

تقييم تردد الطفرات للجينين *p53* و ***K-ras*** في النساء العراقيات المصابات بسرطان المبيض

ميساء غازي جمعة

كلية الطب – جامعة ميسان ، ميسان ، العراق

الخلاصة: يمثل سرطان المبيض رابع نوع من بين السرطانات الأكثر شيوعا التي تصيب الإناث، والسبب الرئيسي للوفاة في العالم تم في الآونة الأخيرة تصنيف أورام المبيض على نطاق واسع إلى مجموعتين متميزتين من النواحي النسيجية والسريرية والجزيئية. النوع الأول الأورام التي تكون فيها الطفرات الجسدية للجينات *BRAF* و *K-ras* هي الاكثر شيوعا، والتي قد تكون لها آثار علاجية مهمة . النوع الثاني الأورام التي تظهر مستويات عالية من عدم الاستقرار الجيني مع قليل من الطفرات الشائعة، بالاضافة الى *P53*، الذي يظهر تغاير في أكثر من 90٪ من الحالات .في هذه الدراسة تم تحليل 58 من سرطان المبيض المشخصة حديثا وذلك للكشف عن البروتين وتواتر الطفرات الجينية للجيني *p53* و *K-ras* في مرضى سرطان المبيض، بالاضافة الى 15عينة من النساء الأصحاء تم استخدامها كمجموعة سيطرة. واستند التحليل على التضخيم باستخدام تقنية تفاعلات السلسلة البوليمرية PCR للاكسونات 5 و 7 من الجين *P53* وكودون 12 من *K-ras*. لم تظهر أيا من عينات السيطرة طفرة في تلك الجينات في حين تم الكشف عن الطفرة في جين *p53* في 13(22.4%)من عينات سرطان المبيض، الذي اظهر فروقات معنوية مقارنة مع الاصحاء)($p<0.05$). أظهرت النتائج أنه من أصل ثلاثة عشر عينة طافرة، كانت الطفرة في اكسون 5 هي الاكثر شيوعا الأكثر شيوعا (% 76.9)10 ثم يليها اكسون-7 في 3(23.07%) من الحالات. إحصائيا لا توجد فروقات معنوية عالية في معدلات الطفرة للجين p53 مع عمر المرضى، حالة انقطاع الطمث، انواع الورم النسيجية، ومراحل الورم المختلفة، تم الكشف عن الطفرة في جين *K-ras* في (5.17%)3 من عينات سرطان المبيض، إحصائيا لا توجد فروقات معنوية عالية في معدلات الطفرة للجين *K-ras* مع عمر المرضى، حالة انقطاع الطمث، انواع الورم النسيجية، والمراحل المختلفة للورم، ولكن كانت العينات الثلاثة الطافرة جميعها ضمن المرحلة الأولى للمرض.من أصل 58 عينة اظهرت عينة واحده فقط طفرة في كلا الجينين. تظهر نتائج الدراسة الحالية أن الطفرات في جين P53 ليست من الأحداث النادرة في أورام المبيض، كما وان الطفرة في جين *K-ras* قد لا يمكن اعتبارها عاملا في التنبوء والتشخيص لسرطان المبيض.

Introduction

Ovarian cancer belongs to the five leading causes of tumor mortality in women in developed countries (1). Approximately 70% of epithelial ovarian cancers are detected at an advanced stage, mainly due to the lack of reliable screening methods. Consequently, there is an urgent need to identify novel diagnostic, prognostic, and predictive biomarkers for development of improved personalized therapeutic regimens for ovarian cancer patients. Most human malignancies are the end result of an accumulation of mutations within tumor-suppressor genes and oncogenes as well as of the dysregulation of specific genes resulting in the antiapoptotic proteins eliminations(2). Molecular studies have identified several genetic alterations such as *p53*, *KRAS*, and *BRCA*1 mutations in ovarian tumors (3, 4).

The *P53* gene is a multifunctional tumor suppressor that is often altered in ovarian and other cancers.(5,6) The *p53* gene encodes a zinc-binding protein with sequence-specific transcriptional activity and (7), exonuclease activity.(8). *p53* normally interacts with a variety of proteins involved in transcriptional regulation, DNA repair, cell-cycle progression, apoptosis, and proteosome-mediated protein degradation. (7,9) Although the biologic and clinical roles that normal and altered *p53* play in cancer remain areas of intense investigation and debate, a number of studies have shown that alterations in *p53* are either associated with or not associated with patient outcomes, such as response to therapy or survival.(6,10). During cancer development, *p53* can be altered by mutation, loss, or silencing of the *p53* gene as well as by transcriptional or posttranscriptional mechanisms.

Studies by the Gynecologic Oncology Group (GOG) and others have indicated that overexpression of *p53* protein, which presumably reflects the presence of a missense mutation, is associated with somewhat worse survival in advanced ovarian cancers.(11,12) It is clear that the frequency of overexpression is significantly higher in advanced-stage III/IV disease (40% to 60%) compared with stage I disease (10% to 20%). Some have interpreted the higher frequency of *p53* overexpression in advanced stage patients as indicative of this being a late event in ovarian carcinogenesis. On the other hand it has been found that *p53* mutated in approximately 40–80% of epithelial ovarian cancers (6,13). In a previous study of 105 ovarian cancer patients, mutations were found in 57% of the cases (14). In the presence of intact *p53*, chemotherapy is followed by growth arrest and the opportunity for DNA repair. However, if repair is sensed to be inadequate, *p53* may activate an apoptotic pathway. Cancers that lack functional *p53* will likely vary in their ability to use alternative pathways to inhibit cell-cycle progression to allow repair of DNA damage or to undergo chemotherapy-induced apoptosis. Furthermore, cancers with functionally inactive *p53* may not only be resistant to chemotherapy-induced apoptosis, but they may also exhibit a more aggressive phenotype because of an altered ability to repair mutations in genes required to prevent or promote ovarian cancer progression.

The *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene encodes the K-Ras protein, an important

component of the tyrosine kinase signaling RAS/MAPK pathway. The K-Ras protein functions as a binary switch, binding GDP in its inactive state and GTP in the active, signal-emitting, state. To inactivate itself, the K-Ras protein interacts with GTPase-activating proteins (GAPs) and, when bound to GDP, it is not able to transmit signals to the cell nucleus. Missense point mutations in the *KRAS* gene abolish the GTPase function and, hence, lead to a constitutively activated protein that cannot turn itself off (15). *KRAS* mutations, most commonly affecting codons 12 and 13, have been described in different types of solid tumors(16). Activation of *RAS* oncogenes also occurs in ovarian tumors. Some studies have shown that *KRAS* mutations are more frequent in mucinous than in nonmucinous neoplasm (17,18,19), whereas other studies have not revealed correlation with histological type (20). All the reported studies are based on a relatively small number of patients and therefore, the results remain a subject of debate. In this study, we analyzed the presence of mutations at exons 5 and 7 of *p53* as well as codon 12 of the *KRAS* gene in 58 ovarian tumors by using conventional polymerase chain reaction and we evaluated whether such alterations correlated with the selected clinicopathological parameters of the patients.

Materials and Methods

Patients and clinical samples: the blood samples from 58 patients with different stages of newly diagnosed ovarian cancer were provided by certain Iraqi hospitals (Al-Kadhemia , AL - Yarmouk Teaching Hospital, Baghdad Hospital) from May 2010- June 2011. All patients underwent their medical history and had undergone clinical and ultrasound examination of the pelvic organs before they were qualified for the study. Fifteen blood samples from healthy donors were used as a control in this study. each case, 5 mL of peripheral blood was collected into an EDTA-containing tube, The samples were stored at -20C^{o} until further processing.

DNA extraction quantification, purity measurement and Electrophoretic analysis

DNA was extracted from frozen blood samples by the gSYNC DNA Mini method (Geneaid/ Taiwan) according to the “frozen blood protocol” .We extracted the DNA from 200 µl of blood in each case. DNA was quantified to measure total DNA concentration by adding 5 µl of DNA to 495 µl of TE buffer, mixing well, and measuring the optical density at wave length 260 nm (21). A calibrated Eppendorf spectrophotometer was used. Total DNA yield was then calculated. The purity of genomic DNA was evaluated on the basis of UV absorption ratio at 260/280 nm. Pure prepreation of DNA had a ratio around 1.8. DNA extracts were analyzed on 2% agarose gels (Fig.1). The gels contained 0.5µg/mL ethidium bromide and were run for one hour at 80V. A 100 bp DNA ladder (Promega/USA) which yielded 10

bands, was used as a ladder. The DNA extract were mixed with loading buffer (Promega/USA). Digital images of the gels were viewed and captured.

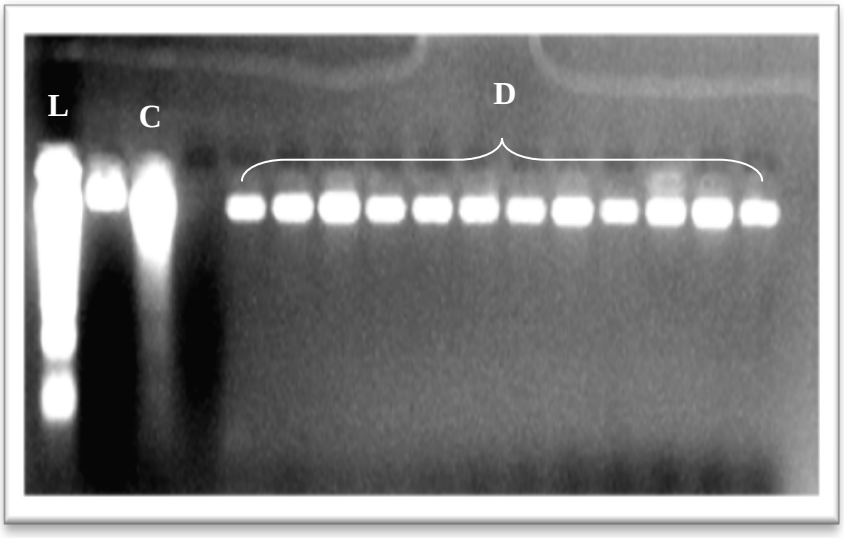

Figure 1:Example of DNA extraction product purification in 2% agarose gel. L:100 bp DNA ladder, C: bands of control DNA, D: bands of DNA extraction yield

Polymerase chain reaction

Polymerase chain reaction (PCR) of exons 5 and 7 of *p53* and codon 12 of *KRAS* were performed using an ABI thermal cycler (Applied Biosystems / Korea). The primer sequences used are shown in Table 1, (Alpha DNA/Canada). For *p53*exons (5 and 7) PCR amplification was performed in a total volume of 25 µl containing extracted DNA, 5 µl dNTPs, 1 µl of each primer, master mix (Promega/USA),12.5 µl, and nuclease free water 5.5 µl. The following program was used : 30 cycles of 94 °C for 2 min, 55 °C for 2 min followed 72 °C for 3. For *KRAS*, the thermal cycling began with denaturation at 94 °C for 1min, 52 °C for 1min, and 68 °C for 2 min. The PCR amplification products were separated by 2% agarose gel electrophoresis and visualized by exposure to ultraviolet light after ethidium bromide staining.

Table 1: Primers used in polymerase chain reaction (Alpha DNA/Canada)

Primer	Sequence
P53-5-F	5'-TTCCTCTTCCTACAGTACTC-3'
P53-5-R	5'-GCCCCAGCTGCTCACCATCG-3'
P53-7-F	5'-CTTGCCACAGGTCTCCCCAA-3'
P53-7-R	5'-AGGGGTCAGCGGCAAGCAGA-3'
KRAS -F	5'-GGTGGAGTATTTGATAGTGTA-3'
KRAS -R	5'-GGTCCTGCACCAGTAATATGCA-3'

Statistical analysis

The overall frequency of *p53* exons and *KRAS* mutations were computed for all 58 cases with respect to age at diagnosis, family history of ovarian cancer, menopausal state, tumor histopathological type, and tumor stages. Differences in proportions were evaluated using the Z-test for proportions comparing.

Results

Samples of the present study were classified in different categories according to certain patient characteristics (family history, menopausal state) tumor histological type, tumor stages, and frequency of mutations as shown in Table 2. The patients mean age 48.2±35 years. The results of the present study showed that , out of the 58 samples examined, 8(13.8%) samples have positive family history while 50(86.2%) samples showed negative family history to cancer diseases. According to menopausal state 34(58.6%) of patients were premenopausal and 24(41.4%) were postmenopausal. According to tumor histological type, epithelial ovarian tumors represented 48(82.75%) of cases which included several subtypes [Serous tumor 22(45.83%), mucinous tumors 19(39.58%), endometriod tumors 4(8.33%), clear cell tumor 2(4.16%), and burner tumors 1(2.08%)], sex cord tumor were 7(12.06%) of cases, and germ cell tumor represented 3(5.1%) of cases.

For *p53* gene, none of healthy control exhibited mutation in that gene neither in exon-5,nor in exon-7, while *p53* mutations detected in 13(22.4%) of ovarian cancer samples, which was significantly higher in compare with healthy controls (p value=0.0739 <0.05). The results showed that out of thirteen mutant ovarian cancer samples, exon-5 mutation was the most frequent and detected in 10(76.9 %), (Fig. 2), followed by exon-7 that detected only in 3(23.07%) of cases, (Fig. 3). In the 13 mutant ovarian cancer samples, several histological types were represented, serous 5(38.46%), clear cell 2(15.38%), endometriod 1(7.69%) and mucinous 1(7.69%) tumors, sex cord stromal tumor 3(23%), and germ cell tumor 1(7.69%). For cancer stages, *p53* mutation detected in 10 (76.9%) patients with stage I, 2(15.8%) patients with stage II, and only one patient (7.7%) with stage III. Statistically there were no significant differences in mutational rates of *p53* gene in patients with age, menopausal state, tumor histological subtypes, and different FIGO stages, but a trend towards a higher mutational rate could be seen in FIGO stage I for both exons.

For *KRAS* gene, none of healthy controls exhibited mutation in that gene, while mutation detected in only 3(5.17%) of ovarian cancer samples(Fig. 4). The histological types of mutant samples represented were, serous tumors 1(33.33%), clear cell tumors 1(33.33%), and mucinous tumors 1(33.33%) There were no significant difference in mutational rates of *KRAS* gene in patients with age, menopausal state, tumor histological subtypes, and different FIGO stages, but all these three mutant samples with stage I. Out of 58 samples only one patient 1(1.7%) have been identified with mutations in both genes.

Figure 2 - Polymerase chain reaction analysis for p53-exons 5 mutation in ovarian cancer samples. L:100 bp DNA ladder, C: band of control, +: band of mutant tumor samples, - : band of un mutant tumor samples

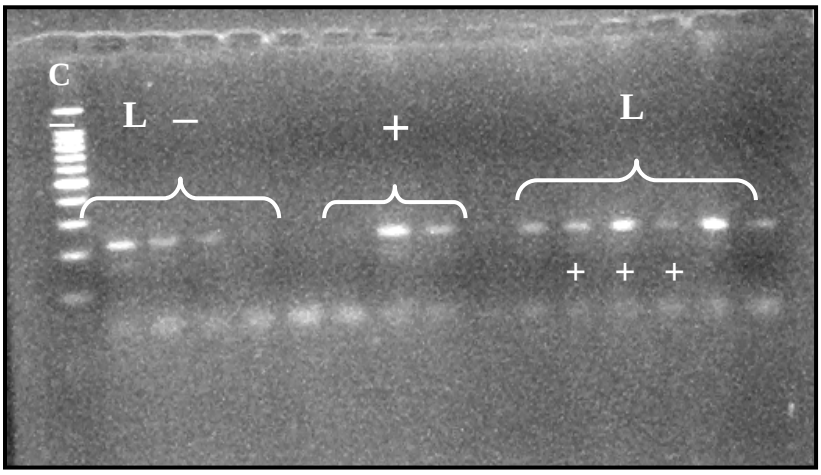

Table 2: Patient characteristics, type of tumor, stage of tumor, and frequency of mutations

Characteristics	All cases	*P53* mutation	*KRAS* mutations
	58	13(22.4%)	3(5.17%)
Family history Yes	8(13.8%)	1(7.7%)	0
No	50(86.2%)	12(92.3%)	3(100%)
Menopausal state Premenopausal	34	7(53.8%)	2(66.66%)
Postmenopausal	24	6(46.2%)	1(33.33%)
Ovarian tumor type Epithelial tumors	48	9(69.3%)	3(100%)
Sex cord tumor	7	3(23%)	0
Germ cell tumor	3	1(7.7%)	0
Tumor stages Stage I	40	10(76.9%)	3(100%)
Stage II	7	2(15.38%)	0
Stage III	11	1(7.7%)	0

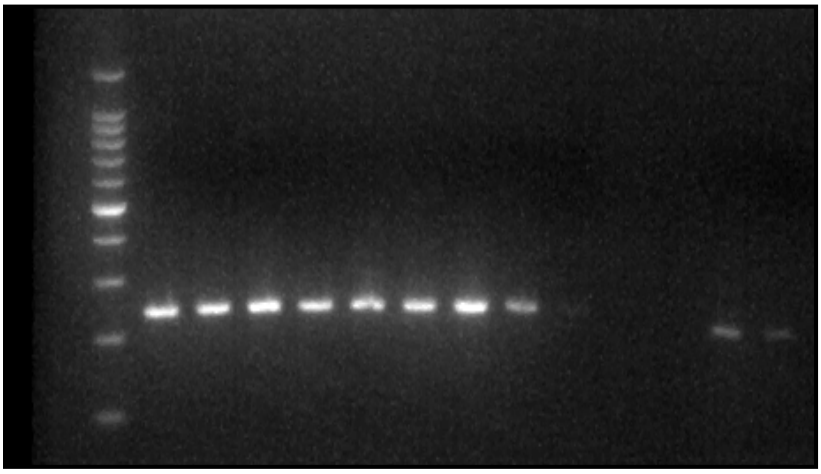

Figure 3 - Polymerase chain reaction analysis for p53-exons 7 mutation in ovarian cancer samples. L:100 bp DNA ladder, C: band of control, +: band of mutant tumor samples, - : band of un mutant tumor samples

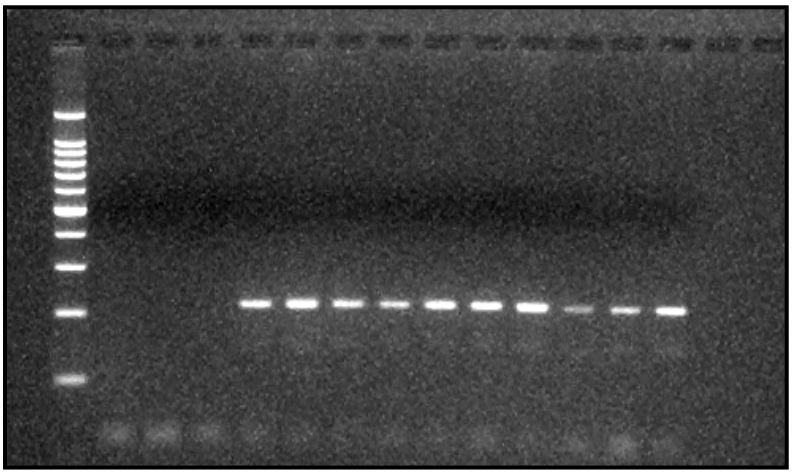

Figure 4 - Polymerase chain reaction analysis for KRAS gene mutation in ovarian cancer samples. L:100 bp DNA ladder, C: band of control, +: band of mutant tumor samples, - : band of un mutant tumor samples

Discussion

Epithelial ovarian cancer is a highly heterogeneous disease with divergent clinical behavior. This heterogeneity is not only reflected in the occurrence of different histological subtypes, but also in the tumourigenetic pathways (22,23,24). In the present study, the incidence of *p53* and *kras* gene mutations was determined in a series of ovarian cancer samples in related with tumor histological types, tumor stages and certain of patients characteristics.

Genetic aberrations affecting the *p53* gene locus in cancer patients have been

extensively studied since the characterization of this gene as a tumor suppressor. *p53* is the most studied tumor suppressor, and mutations in the *p53* gene and subsequent gene product have been related to most cancer types. Aberrant *p53* has been detected in approximately 50% of all invasive epithelial ovarian cancers (25). De Graeff *et al.* (26) determined a prognostic value of *p53* in ovarian cancer through a meta-analysis of 62 previously published studies using a total of 9448 patients. The present study examined the frequency of *p53* gene mutations in peripheral blood of ovarian cancer patients using conventional PCR amplification of exons-5 and 7. The results demonstrated that *p53* mutations detected in 13(22.4%) of ovarian cancer samples, while none of healthy controls exhibited mutation in that gene neither in exon-5,nor in exon-7, these findings similar to that reported by other studies including Angelopoulou *et al*. (27) who detected *p53* mutations in only 14% (8 of 56) of the tested samples, Lianidou *et al*. (28) who detected *p53* mutations in 20% (18 of 89) of ovarian tumor samples, Niwa *et al*. (29) who detected *p53* mutations in only 26% (14 of 54) of ovarian cancer samples, and Teneriello *et al*.(30) who detected *p53* mutations in 20% (9 of 63) of low malignant potential tumors of the ovary, and ovarian carcinomas. On the other hand, the present study results were different from results reported by other studies including Yemelyanova *et al*.(31) who found *p53* mutations in 63% (36 of 57) of the ovarian tumor samples, Shahin *et al*.(32) who found by sequencing that *p53* mutations detected in 57.3% (98 of 171) of ovarian carcinomas, Havrilesky *et al*.(33) who found *p53* mutations in 77% (84 of 125) of the ovarian tumor samples, and Reles *et al*.(34) who found *p53* mutations in 56% (99 of 178) of samples, Laframboise *et al*.(35) who found *p53* mutations in 53% of cases.

The results showed that exon-5 mutation was the most frequent mutation and detected in 10(76.9 %), followed by exon-7 that detected only in 3(23.07%) of cases. These results are in agreement with other studies that reported that mutations in exon-5 may play an important role in the clinical outcome of ovarian cancer since *p53* exon-5 was the target in the vast majority of the cases, including Havrilesky *et al*. (33), Reles *et al*. (34), and Angelopoulou *et al*. (27). Statistically the present study showed no significant differences in mutational rates of *p53* gene in patients with age, menopausal state, tumor histological subtypes, and different FIGO stages, but a trend towards a higher mutational rate could be seen in serous tumors serous 5(38.46%), and FIGO stage I 10(76.9%). These results are similar to that reported by Reles *et al*. (34) and Kappes *et al*.(36) who showed that that *p53* mutations are very frequent in serous papillary carcinomas, particularly in tumors of high grade.

Few studies have investigated the prognostic value of *KRAS* mutation status in ovarian cancer. The results demonstrate a frequency of *KRAS* mutations in only 3(5.17%) of ovarian cancer samples and none of healthy controls. These results are similar to those reported by Dobrzycka *et al*.(37) who detected *KRAS* gene mutations in 6.2% (4 of 64) cases with ovarian carcinomas. Other studies showed slightly higher percentages of mutation frequency including, Nodin *et al*.(38) showed that 17 (11.1%) of ovarian cancer cases harboured mutations in the *KRAS* gene, Auner *et al*.(39) who

detected *KRAS* mutations in 58 (15%) samples deriving from malignant ovarian tissue, Nakayama *et al*.(40) who detected *KRAS* mutations in 8 (13.7%) of ovarian cancer samples, Fabjani *et al*.(41) who detected *KRAS* gene mutations in 20% (17 of 85) cases with ovarian carcinomas, Sieben *et al*.(42) who detected *KRAS* gene mutations in 15% (17 of 113) cases with ovarian carcinomas.

Since the size of mutant samples was relatively small, statistically the present study showed no significant differences in mutational rates of *p53* gene in patients with age, menopausal state, tumor histological subtypes, and different FIGO stages, but a trend towards a higher mutational rate could be seen in FIGO stage I. These results showed some similarities to those reported by previous studies including Nodin *et al*.(38) who found that *KRAS* mutation was significantly associated with lower grade, mucinous histological subtype while no associations were found with age and clinical stage, Dobrzycka *et al*.(37) who did not correlate *KRAS* mutations with the malignant potentials (*e.g.* stage and grade) and patients' age but he detected that there was a tendency towards a higher incidence of *KRAS* mutations in the mucinous. Auner *et al*.(39) found that no significant difference in mutational rates in patients with different FIGO stages, but a trend towards a higher mutational rate could be seen in FIGO stage I tumors, he also detected that. mucinous lesions displayed mutations most frequently. Nakayama *et al*.(40) found that no significant correlation between *KRAS* mutations and the patient's age while mutation is correlated significantly with FIGO stage I, II, pathological grade, and histological subtype, and Fabjani *et al*.(41) who found that *KRAS* mutation status was not correlated with either FIGO stage or histologic type.

ovarian cancer is a group of distinct disease entities with different molecular profiles. In hereditary cancer syndromes, the defective genes are closely associated with cell cycle control and DNA repair, examples being *BRCA1* and *BRCA2* in hereditary breast-ovarian cancer syndrome (43). In sporadic ovarian cancers, however, the most prevailing genetic alterations known are mutations or loss of heterozygosity in the *TP53* gene and/or sporadic mutations or epigenetic silencing of the *BRCA1* gene (44). Mutations of *TP53* can be found in 51–93% of high-grade serous carcinomas, while they are rare in clear cell carcinomas as well as in low-grade serous, mucinous and endometrioid carcinomas (45). In contrast, low-grade serous ovarian carcinomas harbour alterations in *KRAS*, *BRAF* and/or *HER-2* genes, implying different routes of carcinogenesis between high- and low-grade serous types of ovarian cancer (46).

The result of the present study demonstrated the lacking of p53 and *KRAS* mutations in healthy controls, the results also detected that the incidence of p53 mutation frequencies seem to be highly related to tumor histological type of ovarian cancer since the frequency of mutation in epithelial ovarian tumors was higher (69.3%) than those of sex cord and germ cell tumors, there was also a tendency towards a higher incidence of *p53* mutation in the serous tumors (38.46%) than in other histological subtypes of epithelia; ovarian tumors, these result support the previous findings that found that *p53* mutations are strongly associated with

serous carcinomas (47). The results also showed that most of patients harbored mutation 10(76.9%) were with FIGO stage I of disease, these findings may be support the hypothesis that a mutation leading to genetic instability, such as *P53*, that occurred early would predispose cells to other mutations, and rapid progression to a metastatic phenotype, as seen in high-grade malignancies (48).

For *K-ras* gene mutation the results detected that the incidence of *KRAS* mutation frequencies seem to be highly related to tumor histological type of ovarian cancer since all the mutant samples were belong to the epithelial ovarian tumors 3(100%), but there was no correlation with histological subtypes since each one of three cases came with different subtype and the size of mutant samples was relatively small to show significant differences. The results also showed that all three patients who harbored mutation 3(100%) were with FIGO stage I of disease. Few previous studies have determined the prognostic role of *KRAS* alteration in ovarian cancer (49). Several studies found no correlation between *K-ras* gene mutations and survival (50).

In conclusion, the present study results showed that mutations of the *p53* gene are not rare events in ovarian tumors. The majority of mutant samples were harbored mutations, that localized in exon-5. These data suggest that mutations in exon-5 of the *p53* may play an important role in the clinical outcome of ovarian cancer. On the other hand a lower incidence of *K-ras* mutation in ovarian cancer was observed in this study, and also in a previous studies, indicated that *KRAS* mutation status is not a prognostic factor in ovarian carcinomas, and larger cohort of ovarian carcinomas may be required to confirm these findings.

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