ORIGINAL ARTICLE

PATTERN OF KRAS GENE EXPRESSION IN IRAQI WOMEN OVARIAN CARCINOMA

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ABSTRACT

The aim: The goal of this study was to detect if KRAS gene and levels of had any clinical significance in the ovarian cancer by measuring levels of KRAS mRNA.

Materials and methods: The investigation was conducted on 84 tissue samples from newly diagnosed patients with ovarian cancer. Twenty-eight tissue sections with benign ovarian tumors were used as a control group. The qRT- PCR technique was used for measuring and analyzing levels of KRAS mRNA.

Results: Relative increasing of KRAS mRNA level in cancer samples was statistically significant ($P<0.01$) when compared to benign tumors. Statistically no significant differences were found between KRAS mRNA levels and menopausal status or family history. Gene expression has been substantially connected with age groups as the highest levels of KRAS mRNA was recorded in patients with age 50-74 years ($P<0.01$). Endometrium tumors exhibited significant correlations ($P<0.01$) across histopathological tumor types. In correlation with tumor stages, stage I was substantially linked compared to stage I ($P<0.01$).

Conclusions: It was concluded that over expression of the KRAS gene is linked to early stages of ovarian cancer, which implying that mRNA levels could be used as a diagnostic and predictive factor for ovarian cancer. More research with larger groups of ovarian cancer specimens in both primary and advanced stages is needed.

KEY WORDS: Ovarian cancer, CA ovary, KRAS, KRAS mRNA, Gene Expression

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INTRODUCTION

In women, ovarian cancer represented the fifth leading cause of death causes related with cancer. It has been estimated that the survival rate is lower than fifty percent 30%-40% only [1]. The incidence ratio of epithelial ovarian tumor is one for each 72 women [2-3], with increasing rate in developing countries. Three categories of ovarian tumors have been identified: epithelial 60%, germ cell 30%, and sex-cord stromal 8%, the beginning of malignant ovarian carcinoma in vast majority of cases 80-85%, occurs in ovarian epithelium [4-5]. Absence of symptoms make it difficult to diagnose epithelial ovarian tumors during early stages, the asymptomatic condition is due to certain factors that related to clinical and histopathological features [6]. Losing of portent lesions and their evolution, is the reason why vast majority of patients with ovarian tumors are diagnosed at advanced stages with poor prognosis [7-8]. Over the years many genes/proteins have been identified as fundamental genes in carcinogenesis as they play a key role in the development and progression of various cancer types. RAS genes and their products are among the key genes that frequently mutate in human cancers which make them difficult to be targets for cancer therapy [9]. KRAS is a member of the RAS GTPase family. This gene involved in cells proliferation and apoptosis among variety of biological processes including [10]. Genetic variation in the exons of KRAS gene may associate with protein hyperactivity. Increasing activity of protein is an oncogenic mutation that very common human cancer. This sort of mutations was detected in a variety of human cancers including 17% of lung cancer, and 14% of ovarian cancer [11]. Previous studies have been providing substantial information that clarifies the role of KRAS in ovarian physiology and pathology, one of that information that the activation of RAS genes is regulated within ovarian surface epithelial cells as well as granulose cells. This activation is definitive for initiation of luteinizing hormone pathway which is involved ovulation. As a result any genetic variation that leading to mutation or over expression in the active form of KRAS gene leads to interception of granulose cell cycle and then deteriorate follicle growth [12]. On the other hand, over expression of KRAS gene in ovarian surface epithelial cells that have deficiency with Pten gene may responsible for the development of serous epithelial adenocarcinoma. Other studies reported that the over expression of mutant KRAS increase the cells susceptibility to tumorigenesis and stimulate senescence of certain ovarian cells particularly epithelial and fibroblast cells [13]. Goal of this study is to detect if the KRAS gene and its mRNA expression levels in ovarian cancer patients had any clinical significance.

THE AIM

The goal of this study was to detect if KRAS gene and levels of had any clinical significance in the ovarian cancer by measuring levels of KRAS mRNA.

MATERIALS AND METHODS

To implement the present study, tissue sections from eighty four patients who already diagnosed with ovarian carcinoma at different stages, have been used. Tissue biopsies are obtained from certain Iraqi hospitals after the patients undergo any of the following surgeries: Total abdominal hysterectomy with bilateral salpangio opherectomy (TAH-BSO), subtotal abdominal hysterectomy, vaginal hysterectomy, and endometrial. For a control 28 tissue sections of benign tumors were included. Tissue sections then maintained and subjected to molecular analysis. Demographic and pathological information were collected from hospitals records.

RNA EXTRACTION, REVERSE TRANSCRIPTION

Formalin fixed paraffin-embedded tissue sections were subjected to sectioning and then RNA extraction using RN easy FFPE kit for RNA purification (Qiagen – USA). Total RNA has been reversed using the reverse transcription Thermo-Script TM (Invitrogen/United States) kit. This procedure was conducted in the 50 µl reaction volume composed of 15 µl denaturized RNA,0.2 µl Random hexamere primers 3µg/µl, 5µl of 10 µldNTP Mix, 10µl of 5x cDNA synthesis buffer, 2.5µl RNase OUT (40U/µl), 2.5µl Thermo Script RT (15 units/µl), 14.8µl DEPC-treated water. The samples were then placed in a 96-pit thermal cycler, cycling at 25°C for 10 minutes, 10 minutes at 37°C, 60 minutes at 42°C, followed by 75°C for five minutes. The converted cDNA has been stored at -80°C and used to amplify Kras as a PCR template.

QUANTITATIVE REAL-TIME PCR

Real-time PCR was performed using the SYBR Green Supermix Kit (Qiagen – USA), Krasprimers that described previously by Latif AH [14]., were utilized in this study, for PGK1 primers were forward:

GCGTCAAGGTGAAGATAATACCTAA,

And reverse:

CATTTAAACTTGTGGTTGCTCTT

The Applied Biosystems 7900 equipment was used to run quantitative real-time PCR tests in duplicate. Reaction volume of 20 µl was used for PCR reaction. It was contain of SYBR Green master mix (10 µl), primer mixes (1 µl), RNase free water (5 µl0, and cDNA template (4 µl). Conditions of Real-Time PCR was as follows: stage 1: 50 °C for 2 min., stage 2: 95 °C for 10 min., stage 3: a two-step cycle process (95 °C for 15 sec. and 65 °C for 1 min.) repeated for 6 cycles, and stage 4 in a two-step cycle procedure (95°C for 15 Sec. and annealing 61°C for 1 min.) repeated for 40 cycles. The endogenous reference gene (*PGK1*) was used as a control gene.

DATA ANALYSIS

Results of RT-PCR analyzed using 2−ΔΔCt method [15]. Comparative standard curves were used to assess the efficiency (E) of qPCR amplification for target and reference genes, figure (1). Serial dilutions of reaction materials that included cDNA of reference sample and study primers were used for standard curve generation. Ct values against logarithmic values plotted for estimation proportional of cDNA copy numbers in the reference sample and stability and efficiency of primers. Slope and equations of effectiveness (E) were used as following:

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

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

The cycle's threshold (Ct) is defined for each sample as the number of PCR cycles required for the fluorescence defined by the user. The Ct is inversely proportional to both the target (Kras) and reference (PGK1) genes. The level of Kras mRNA was determined by the following equations:

Logarithmic copy no.$_{(PGK1)}$ = (Ct-32.85) /-3.3592

Copy no.$_{(PGK1)}$ = 10^Log copy

Logarithmic copy no (Kras) = (Ct- 37.437) /- 4.4513

Copy no (Kras) = 10^Log copy

Fold change = copy no. ((Kras)/copy no.$_{(PGK1)}$)

STATISTICAL ANALYSIS

The SAS (2012) program for the Statistical Analysis System was employed to determine the impact on study parameters of various variables. The smallest difference – LSD (ANOVA Variation Analysis) or T-Test – has been used in this research in order to compare the means.

RESULTS

Eighty four samples with ovarian cancer and 24 samples with benign ovarian tumors that used as a control group were studied for the expression Kras. The median age was 47 years old and the patients were 14-70 years old. All samples of ovarian cancer have been negative for family history, according to the family's history. The clinical features of ovary cancer samples are listed in table (I). With respect to menopause, 36(46.1%) of the samples were premenopausal, while 42(53.9%) of these were postmenopausal. According to the International Federation of Gynecology and Obstetrics (FIGO) surgical staging system, the majority of samples 57(73%) were in stage I, while the other 21(27%) were at stage III. The samples have been divided into five clinical groups according to tumor histology types; Serous epithelial tumors 33(42.3%) samples, mucinous epithelial tumors 15(19.2%) samples Endometriod tumors 21(26.9%) samples, germ cell tumors 6(7.695%) samples and 3(3.84%) of Burner tumors. Table (II) shows that in both malignant and benign tumor Kras mRNA was expressed. In ovarian cancer samples the highest Kras mRNA levels were statistically important in comparison to benign tumor (mean ± SE: 661, 51 ± 75, 09, **P≤0.01), respectively (mean ± SE: 7, 82 ± 0.22). The present study shows statistically no significant differences in gene expression levels with menopause and family history, while significantly associated with age groups, since the highest levels of gene expression in patients aged between 50-74 years (mean ± SE: 1445.96 ± 82.37,**P≤0.01). In correlation with the histopathological type of ovarian tumors Endometriod tumors showed statistically significant difference in the level of Krasgene expression (Mean ± SE: 2469.55 ± 137.06, **P≤0.01)

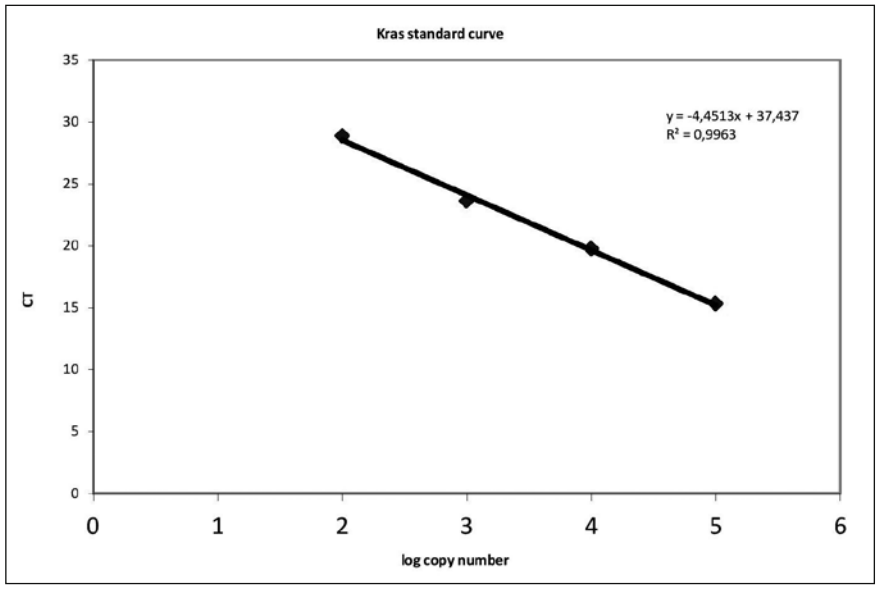

Fig. 1. Standard curves for qPCR efficiency calculation of target Kras gene

Table I. Clincopathological characteristics of patients with ovarian carcinoma

Age groups	
0-14 years (Children)	9(10.7%)
15-24 years (Teenagers and young adults)	3(3.57%)
25-49 years (Adults)	33(39.28%)
50-74 years (Old age)	39 (46.42%)
State of menopause	
Premenopausal no. (%)	40(47.6%)
Postmenopausal no. (%)	44(52.4%)
Family history	
Positive no. (%)	0
Negative no. (%)	84(100%)
Tumor histological types	
Serous epithelial tumors	34(40.47%)
Mucinous epithelial tumors	20(23.8%)
Endometriod tumors	21(25%)
Germ cell tumors	6(7.14%)
FIGO stages	
Stage I no. (%)	63(75%)
Stage III no. (%)	21(25%)
Burner tumours	3(3,57%

compared with other histopathological tumor type. Statistic difference between the 57(73%) stage I samples with the highest expression level (Mean ± SE: SE: 831.78 ± 71.55, **P≤0.01) and the 21(27%) stage III samples (Mean ± SE: 59.45 ± 3.16) were observed according to the tumors stage.

DISCUSSION

A link between RAS family genetic variation like mutation or over expression and tumor lesions with advanced degree, have been recorded by only a few studies. In the present study, relative KRAS gene expression levels were evaluated in 84ovarian tissues obtained from patients with benign and malignant tumors using the real-time PCR method. Ovarian cancer samples in comparison to benign tumor. Similarly to this result, Hong et al [16] reported that there was almost no p21Ras expression in normal breast tissue, but high level of expression in breast cancers. Latif [14] also detected that the mRNA of KRAS gene was highly expressed in samples of colorectal cancer comparing with controls. In correlation with clincopathological parameters, the present study shows that the relative levels of KRAS mRNA and older age of patients were significantly associated. Endometriod tumor histological type and primary stage of tumor (stage I). Except for the age of patients, Pzik et al. [17] found that none of the clincopathological parameters they looked at (patients' sex, age, and tumor histological type, stage, grade, and chemotherapy treatment) were significantly linked to KRAS expression and levels of KRAS mRNA. Wan et al. [18] found significant associations between KRAS expression and cancer patient age, with the highest levels of KRAS mRNA found in patients aged over 56 years, which is similar to the current study's findings. Their findings, on the other hand, contradicted the findings of the current study, as they discovered a significant link between high levels of KRAS mRNA and poor tumor differentiation. The current study's findings contradicted those of Birkeland et al. [19], who discovered that high KRAS mRNA levels were significantly associated with advanced FIGO stage, non-Endometriod histological type, high grade, and lymph node metastasis. What is interesting about the findings of the present study is that the KRAS gene over expression increased remarkably in the subgroup of patients over 50 years of age. Also what is interesting the association of KRAS over expression KRAS with early stage of ovarian cancer which is conflict with results of most previous studies that demonstrated the correlation between KRAS over expression and late stages

Table II. Relative expression levels of KRAS mRNA in ovarian cancer patients, compared to clincopathological features

KRAS gene expression	
Tumor group	Mean ± SE of KRAS gene
Benign ovarian tumors	7.82 ± 0.22
Malignant ovarian tumors	661.51 ± 75.09
T-test	47.911**
** (P≤0.01).	
Age groups	Mean ± SE of KRAS gene
children age 0-14 years	87.46 ± 4.07
Teenagers and young adults aged 15-24 years	5.76 ± 0.63
Adults aged 25-49 years	3.98 ±0.42
Adults aged 50-74 years	1445.96 ± 82.37
LSD Value (P-value)	52.269 **(0.0001)
**P≤0.01	
Histological tumor type	Mean ± SE of KRAS gene
Mucinous	10.48 ± 1.94
Serous	36.58 ± 2.66
Endometriod	2469.55 ± 137.06
Germ cell tumor	35.52 ± 1.83
Burner tumor	2.692 ± 0.39
LSD Value (P-value)	23.605 **(0.0001)
** (P≤0.01)	
Tumor stage	Mean ± SE of KRAS gene
Stage 1	831.78 ±71.55
Stage 3	59.45 ±3.16
LSD Value (P-value)	37.205 **(0.0001)
**P≤0.01	

of tumor. The majority of previous research has focused on the frequency of mutations in the KRAS gene. They claim that the KRAS gene is frequently mutated in various types of tumors and plays a role in cell proliferation, apoptosis, migration, and differentiation. KRAS expression has been studied for its prognostic value in a variety of cancers, but the majority of studies have found a link between KRAS gene over expression and advanced tumor stages. To our knowledge, the present study is one of the few studies that have found a link between KRAS gene over expression and tumor development in the early stages. In general, over expression of Ras genes has been linked to a poor prognosis for colon carcinoma, leading to the conclusion that Ras over expression cannot be used as a predictive factor [20]. On the other hand, another study on the lesions of cervix adenocarcinoma as found that Ras genes were over expressed, but no link between Ras over expression and prognosis was discovered [21].The current study concluded that over expression of the KRAS gene is associated with early stages of ovarian cancer, implying that mRNA levels of this gene could be used as a diagnostic and predictive factor for ovarian cancer. Further studies with a larger cohort of ovarian cancer specimens in both primary and advanced stages are needed to confirm these findings.

CONCLUSIONS

The present study concluded that over expression of the KRAS gene is linked to early stages of ovarian cancer, which implying that mRNA levels could be used as a diagnostic and predictive factor for ovarian cancer. To confirm these findings, more research with larger groups of ovarian cancer specimens in both primary and advanced stages is needed.

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The Author declare no conflict of interest.

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