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Expression of Carbonic Anhydrase IX (*CA9*) in Human Tumor Tissue of Patients with Ovarian Cancer

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Abstract: The aim of the present study is to determine the expression levels of the *CAIX* gene in the Paraffin-embedded tissue blocks from 43 patients with different stages of newly diagnosed ovarian cancer as well as 14 tissue samples of patients with benign ovarian tumors were used as a control group. The study performed using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR). According to malignancy status the percentage of patients with *CAIX*-positive gene expression was significantly higher in compare with benign tumor patients. Depending on the cutoff value, the present study showed that 40(93.02%) of ovarian cancer samples were *CAIX positive*, which is statistically significant comparing with *CAIX*-negative samples 3(6.97%). Statistically there was no significant difference in the levels of gene expression with age, menopausal state, and family history. In correlation with histopathologic tumor types and tumor stage, the present study showed significant associations of high level of *CAIX* gene expression with mucinous histopathologic tumor types($p<0.05$), and stage I of ovarian cancer ($p<0.\ 0.05$). The present study concluded that the possibility of using *CAIX* gene as a useful tool for discriminating malignant ovarian tumors from non-malignant ones. We also demonstrated the diagnostic value of *CAIX* gene for early diagnosis of ovarian cancer.

Key words: carbonic anhydrase IX, CA9, ovarian cancer.

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Introduction

Ovarian cancer was described as the seventh most common cancer among women (1). It is also considered as the fifth most common cause of cancer death and gynecological malignancy leading cause of death in women (2). The mortality rate of ovarian cancer still has relatively high, despite advanced techniques that used for early diagnosis and developing therapeutic strategies. This is may be due to the difficulties facing early detection since many ovarian cancers are asymptomatic, and often the time of diagnosis is associated with the cancer cell dissemination to the peritoneum (3). Many solid tumors including ovarian tumors are produced hypoxia in their microenvironment which due to vasculatures outgrowing for several cubic millimeters. The tumor microenvironment involve many events that are important in carcinogenesis which may also leading for development of a phenotype that could be more aggressive, hypoxia is one of these events that increase proliferation and invasiveness, metastasis formation, and poorer prognosis(4, 5), which in turn increased the resistance of hypoxic malignant cells to chemotherapy and radiotherapy(6). As a result, it has been suggested that the hypoxia act as an

adverse prognostic factor that play a critical role in cancer outcome. The cellular responses to hypoxia is regulate by many factors, one of these hypoxia regulating key factors is carbonic anhydrase IX (CA9) which identified for the first time as a membrane-associated enzyme in the cervical cancer cell line HeLa, that plays a role in pH regulation (7). The acidic extracellular pH and an alkaline intracellular pH of cancer cell can be maintain by *CAIX* induction, that is are critical for both proliferation and invasion of cancer cell (8). The hypoxia in various cancer types may induce elevated levels of *CAIX* that related to poor prognosis (9, 10). Previous studies have been reported that the hypoxia inducible factor (HIF) transcriptional complex regulate the expression of *CAIX* in aberrant oxygen states and acidic conditions (11, 12). In this paper, we will demonstrate the diagnostic and prognostic value of *CAIX* in ovarian cancer.

Materials and Methods

The tissue samples used in this study included 43 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 Misan) after patients underwent to total abdominal hysterectomy and bilateral salpangio-opherectomy (TAH-BSO), subtotal abdominal hysterectomy, vaginal hysterectomy, and endometrial biopsy, 14 samples of patients with benign ovarian tumors tissues were used as a control. Information that related to patients history and tumor properties were obtained from 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 Reverese Transcription and quantitative Real Time PCR at Molecular Oncology Unit in Guy´s hospital – Kings college/ London.

RNA extraction, reverse transcription and real-time RT-PCR assay

The total RNA of ovarian cancer and benign tumor tissues were extracted using the RNeasy FFPE Kit which designed for purifying total RNA from FFPE tissue sections (Qiagen - *USA*) following the protocol provided by the manufacturer. Total RNA was reversely transcribed using Thermo-Script™ Reverse Transcription kit (Invitrogen/ USA). The cDNA synthesis was performed in a reaction volume of 50 µl. All reaction mixtures were prepared on ice. In a 0.5 ml tube, 15 µl of RNA were denaturized by incubating at 65ºC for 5 min and then placed on ice. The 5 x cDNA synthesis buffers was vortex for 5 seconds just prior to use. The master mixture components and their volume are listed in (Table 1). The master reaction mix was added into each reaction tube. The samples were then placed in a 96 Well Thermal Cycler, and cycled at the following conditions: 10 minutes at 25ºC, 10 minutes at 37ºC, 60 minutes at 42ºC followed by 5 minutes at 75ºC. The converted cDNA was stored at -80°C and then used as a template for PCR amplification. Primers were designed by using primer 3 plus software, primers

sequencing of target and endogenous genes listed in Table 2. Quantitative real-time PCR assays were performed in triplicate using the Applied Bio systems 7900 machine. The Real time PCR system primer and SYBR Green master mix were used for quantitative assessment. The amplification reaction was performed in a 20 µl volume containing 10 µl of SYBR Green master mix, 1 µl of primer mixes, 5µl of RNase free water and 4µl of cDNA template. Real-Time PCR protocol was as follows; stage 1: 50ºC for 2 minutes, stage 2: 95ºC for 10 min, stage 3: in a two-step cycle procedure (95ºC for 15 Sec. and 65ºC for 1 min) repeated for 6 cycles and stage 4 in a two-step cycle procedure (95ºC for 15 Sec. and annealing 61ºC for 1 min) repeated for 40 cycles. The estimation of PCR amplification efficiency (E) of a real-time PCR reaction was determined using slope of a standard curves. The efficiency (E) calculation was performed using the following equations:

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

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

The Ct value is used to compare across all samples. The Ct is inversely proportional to the amount of starting mRNA of the target gene (*CAIX)* 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:

$Log\ copy_{\ (endogenous\ control\ gene)} = (Ct - 32.85) / -3.3592$

$Copy\ number_{\ (endogenous\ control\ gene)} = 10^{\wedge}Log\ copy$

$Log\ copy_{\ (CAIX)} = (Ct - 34.82) / -3.5126$

$Copy\ number_{\ (CAIX)} = 10^{\wedge}Log\ copy$

$Fold\ change = Copy\ number_{\ (CAIX)} / Copy\ number_{\ (endogenous\ control\ gene)}$

Table (1): The reaction master mixture for cDNA preparation.

Reagents	**Volumes for 50** (µl)
Denaturized RNA	15
Random hexamere primers 3µg/µl	0.2
10 mM dNTP Mix	5
5x cDNA synthesis buffer	10
RNase OUT (40U/µl)	2.5
ThermoScript RT (15 units/µl)	2.5
DEPC-treated water	14.8
Total	50

Table (2): Primers sequences of target and endogenous genes.

Primer	**Sequence**
CAIX -F	5′- GTGGAAGGCCACCGTTTC -3′
CAIX -R	5′- CTCGTCAACTCTGGCAAAGG -3′
PGK1-F	5′- GGGAAAAGATGCTTCTGGGAA -3′
PGK1-R	5′- TTGGAAAGTGAAGCTCGGAAA -3′

Statistical Analysis

Results of the present study were subjected to statistical analysis using the Statistical Analysis System-SAS, version 2012. The comparison between percentages was determined depending on Chi-square test, while the comparison of mean values depended on least significant difference (LSD).

Results

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 3). 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. In regarding to malignancy status the ovarian cancer patients with *CAIX* -positive gene expression showed the highest levels of *CAIX* expression comparing with benign tumor patients, which was statistically significant (p <0.05). By using the mean value of *CA9* gene expression in benign tumors (22.48) as the cutoff value to separate tumor samples into *CAIX*-positive and *CAIX*-negative, the present study showed that out of 43 samples with ovarian cancer, *CAIX* was positively expressed in 40(93.02%) of samples, which showed statistically significant differences (p <0.01) in compare with *CAIX* -negative samples 3(6.97%). Relationship between *CAIX gene* expression and clinicopathologic parameters are listed in (Table 4). The present study showed no statistically significant differences in the levels of gene expression with age, menopausal state, and family history. In correlation to the histopathologic tumor types, the mucinous ovarian tumors showed statistically significant differences in the level of *CAIX gene* expression comparing with other tumor types (p<0.05). In correlation with tumor stages, samples with stage I showed statistically significant differences in the level of *CAIX gene* expression comparing with stage III, (p<0. 0.05).

Table (3): Clinical features of ovarian cancer samples.

Age groups	n(%)
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(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 (4): Effect of clinicopathological features on *CAIX* gene expression in ovarian cancer patients.

***CAIX* gene expression**	
Tumor group	**Mean ± SE of *CAIX* gene**
Benign tumors	22.48 ± 8.98
Malignant tumors	660.54 ± 76.49
LSD Value P-value	235.64 * 0.052
* (p<0.05)	
Histological tumor type	**Mean ± SE of *CAIX* gene**
Mucinous	2684.58 ± 268.72
Serous	37.72 ± 26.01
Endometriod	120.62 ± 22.65
Steroli-lyding	85.91 ± 24.09
Germ cell tumor	0.213 ± 0.06
Burner tumor	0.002 ± 0.00
Clear cell	0.001 ± 0.00
LSD Value P-value	265.49 * 0.0527
* (p<0.05)	
Tumor stage	**Mean ± SE of *CAIX* gene**
Stage 1	940.36 ± 216.32
Stage 3	25.64 ± 2.18
LSD Value P-value	327.44 * 0.0276
* (p<0.05)	

Discussion

In the present study we examined the possibility of using *CAIX* gene as a diagnostic and prognostic marker depending on the levels of gene expression in ovarian tumor tissues by using qRT-PCR technique. The results of the present study showed that the *CAIX* -positive ovarian cancer showed the highest levels of *CAIX* gene expression, which was significantly higher compared with benign tumor patients, these findings indicates high specificity of *CAIX* gene as a diagnostic marker to discriminate malignant from benign ovarian tumor. The present study also showed that *CAIX* positively expressed in 40(93.02%) of samples, which showed statistically significant differences in compare with *CAIX* -negative samples 3(6.97%). These results have some similarity to that reported by other studies including Baker *et al*. (13) who found that the staining for xenograft was ranging between 90-100 % when *CAIX* expression was measured by IHC using OVCAR-3 human ovarian cell lines, they also reported that the *CAIX* protein expression was observed in primary as well as metastatic cancer frozen tissue samples from all five patient that he used to confirm their results. Kim *et al,* (14) reported that the *CAIX* was detectable in 99 (79.2%) of the 125 specimens while 26 specimens (20.8%) showed negative staining for *CAIX* expression using immunohisto-chemistery technique. Martin *et al*.,(15) reported the *CAIX* gene expression in all the lanes at the expected molecular weight when compared with the house keeping genes. Our result different from that reported Choschzick *et al*.,(16) who found that *CAIX* expression was found in only 37(18%) out of 205 of ovarian cancer specimens they tested. The identification of *CAIX* expression positivity according to the age groups, family history, and menopausal state showed no significant correlation. In regarding to the histopathologic tumor types, the mucinous ovarian tumors showed statistically significant differences in the level of *CAIX gene* expression comparing with other tumor types. The present study results were similar to that reported by Martin *et al*.,(15) who suggested the possibility of using *CAIX* gene as a valuable diagnostic marker for ovarian mucinous tumors, since they all show high levels of *CAIX* expression. Kim *et al,*(14) reported the high significant correlation of *CAIX /GLUT-1* coexpression with histologic type, they showed that the most common type of cancers were mucinous and endometrioid types that positive for coexpression of *CAIX /GLUT-1*. Similarly Choschzick *et al*., (16) reported that the mucinous and endometrioid types of ovarian carcinomas showed the highest levels of *CAIX* expression. In correlation with stages, stage I showed statistically significant difference in the level of expression comparing with stage III. The data of the current study incompatible with most of the previous study which indicated that no significant associated between the high levels of *CAIX* expression and FIGO stages. Kim *et al,*(14) reported that the no correlation was observed between the high coexpression of *CAIX /GLUT-1* and some clinical factors, such as tumor size, tumor grade, tumor necrosis, FIGO stage, and disease recurrence. Choschzick *et al*.,(16) reported that there was no association between the over expression of *CAIX* and tumour

clinical features (stage, grading, and mitotic count) of ovarian carcinomas. Studies that carried out on different cancer types including breast, upper gastrointestinal tract, cervix, ovary, and lung cancer have proved that the poor outcome of tumor was associated with over expression of *CAIX* (16, 17). Previous studies indicated that *CAIX* over expression could be a useful marker for hypoxia and a potential target for diagnosis and treatment of ovarian cancer (18). In general the previous studies demonstrated that the correlation between *CAIX* over expression and clinicopathologic parameters tend to be diverse, depending on the tumors sites, and researchers. On the other hand, the scientist suggested that *CAIX* expression could be an important predictive marker for selection of the most appropriate adjuvant therapy, this importance depending on the fact that tumor hypoxia associated with tumor resistance to chemotherapy and radiotherapy, poor survival and more aggressive phenotype (17). In conclusion our study demonstrated the possibility of detecting of that gene transcript in benign as well as cancer samples but with wide differences in the level of gene expression which in turn reflect the value of *CAIX* gene as a useful tool for discriminating malignant ovarian tumors from non-malignant ones. The present study also indicated the diagnostic value of *CAIX* gene for early diagnosis of ovarian cancer since the level of gene expression was elevated in most of the stage I cancer samples regardless the tumor histological type. The prognostic value of that gene may be not proved in this study due to a small number of samples with stage III, so further studies are recommended using larger number of samples with advanced stages.

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