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Assessment of Co-Infection of Human Cytomegalovirus DNA and Epstein Barr-Virus (ZEBRA-Genes) in Tissues of Ovarian Tumors

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Abstract: Infection by Epstein Barr-Virus (EBV) begins with a short replication phase. The virus remains in a latent phase, only entering the lytic phase in response to a cascade of transcriptional signals. These signals are triggered by the ZEBRA protein (BZFL1) along with Rta (BRLF1). Human cytomegalovirus (HCMV) is responsible for a lifelong persistent infection which ranged from 50% to 90% in adult population, and is related to either socioeconomic status or geographic location. HCMV infection might lead to buildup tumor cells via the protection of certain tumor cells from apoptosis and modulating angiogenesis. The study was designed to determine ZEBRA-EBV gene and DNA-HCMV infections in tissues from ovarian tumors. An 150 ovarian tissues were examined for ZEBRA-EBV gene with DNA-HCMV. Those samples belonged to (45) patients diagnosed with ovarian cancer ; (45) from benign ovarian tumors and (20) patients with borderline ovarian tumors as well as (20) apparently normal ovarian tissues. The detection of ZEBRA-EBV gene and DNA-HCMV were done by chromogenic *In situ* hybridization (CISH). The positive results of ZEBRA - EBV -CISH detection in malignant ovarian tumors, where 64.4% (29 out of 45 tissues) showed positive signals. While ,in the benign ovarian tumors group were 37.8 % (17 out of 45 tissues) ,followed by borderline ovarian tumors & the apparently healthy ovarian control tissues were 30% (6 out of 20 cases) and 7.5% (3 out of 40 cases), respectively. The present study shows the positive results of HCMV-CISH detection in malignant ovarian tumors, where 55.6% (25 out of 45 cases) showed positive signals, while, 44.4% negative signals which represented 20 out of 45 cases in this group. While, in the benign ovarian tumor group was 35.6% (16 out of 45 cases). Negative signals which in benign group represented 29 out of 45 cases constituted 64.4% .Whereas ,the positive results in borderline ovarian tumor group were 40% (8 out of 20 tissues), followed by the apparently healthy ovarian control tissues was 12.5% (5 out of 40 tissues). We concluded from this study, ZEBRA -EBV genes as well as HCMV-DNA positive signals in malignant, borderline and benign tumors tissues ,they suggest an important role for these viruses in the development of ovarian tumors in Iraqi patients.

Keywords: Ovarian Tumors, ZEBRA –EBV*; HCMV, CISH.*

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Introduction:

Ovarian cancer is one of the most lethal gynecological malignances and the fifth leading cause of cancer death in women in women worldwide (1). Ovarian cancer (OC) is the seventh most commonly diagnosed cancer among women in the world and the tenth most common in China (2). Ovarian cancer is the most life-threatening type of gynecological cancer in females. According to Cancer Statistics, there were an estimated 22,440 novel cases of ovarian cancer in 2017(3). In Iraq, ovarian cancer is the

fifth most common cause of death, and the 6th in the list of most common cancers. Since most ovarian cancer patients are diagnosed at late stages, the overall survival rate remains a dismal 30%(4). Although ovarian tumors can arise from three different cell types: epithelial cells, germ cells, and sex cord stromal cells, the vast majority of ovarian cancers are epithelial in nature(5). Even within epithelial ovarian cancers there exist various histologic subtypes and molecular subgroups (5). This has prompted the classification of epithelial ovarian cancers into two groups. Type I tumors, clearly linked to ovarian precursor lesions, encompass all histologic subtypes including low grade serous, endometriod, mucinous, and clear cell carcinomas. They are defined by their slow growth and multiple genetic mutations (6). In contrast, Type II tumors are highly aggressive, confer a much poorer prognosis, and many have been linked to precursors arising from the fallopian tube epithelium (6).

Epstein-Barr virus (EBV) is an enveloped, ubiquitous gamma herpes virus with a double-stranded DNA genome encoding more than 85 gene. Epstein-Barr virus -ZEBRA protein is an immediate-early viral gene of the Epstein–Barr virus (EBV) of the Herpes Virus Family, which induces cancers and infects primarily the B-cells of 95% of the human population. This gene (along with others) produces the expression of other EBV genes in other stages of disease progression, and is involved in converting the virus from the latent to the lytic form (7).

Human cytomegalovirus (HCMV) is a ubiquitous herpes virus that leads to a life-long persistence. HCMV is a virus that commonly infects humans and many other animals. The frequency of infection ranges from 50% to 100% in the general adult population. HCMV has been established as the major cause of viral infection in pregnancy .Infections of the feotus can result due to initial maternal infections, as well as, reinfection and/or reactivation in HIV positive mothers (8).

In recent years, evidence has emerged which indicates that Herpes virus may also have a role in ovarian cancer. Previous study have shown evidence of Epstein–Barr-virus in ovarian cancer (9). Recently, human cytomegalovirus (HCMV) has been detected in ovarian cancer(10). Who evaluated HCMV in ovarian cancer tissue specimens obtained at pre- and post chemo therapytumor resection. Also(11), who examined the prevalence of HCMV in ovarian cancer tissues by polymerase chain reaction .

This study is aiming to analyze the rate of concordance ZEBRA-EBV and HCMV in ovarian tissues from a group of Iraqi female patients with ovarian tumors as well as apparently healthy ovarian tissues.

Patients and Methods:

This study was designed as retrospective case control study. It involved 150 selected formalin fixed, paraffin embedded ovarian tissue blocks that were distributed on the following groups: Forty-five blocks of malignant ovarian tumors, Forty-five blocks of benign ovarian tumors, Twenty blocks of borderline ovarian tumors, Forty blocks of normal ovarian tissues as a

control. The present study include two laboratory techniques: detection of Epstein Barr Virus (ZEBRA -EBV Genes)and Human Cytomegalovirus (HCMV) by Chromogenic *In Situ* Hybridization (CISH) technique. Malignant and benign blocks were collected from the archives of histopathology laboratories of Middle Euphrates Hospitals including AL-Sadar , AL-Hilla , AL-Diwania, AL-Muthana, Baghdad teaching hospitals as well as many private laboratories. The diagnosis of these tissue blocks were based on the obtained pathological records of these tissues from hospital files as well as records of histopathologist private laboratories. Following trimming process of these tissue blocks, a confirmatory histopathological re-examination of each obtained tissue blocks were done by consultant hispathologist.

Proviral Probes (DNA Probes for integrated DNA of *ZEBRA -EBV Gene, HCMV*):

In this method design & request a probes for detection of *ZEBRA -EBV Gene, HCMV* by CISH complementary to a sequence of *ZEBRA -EBV, HCMV,* genes, and design a probe for detection of *ZEBRA -EBV Gene, HCMV* by Zytovision company/Germany.

***ZEBRA -EBV* Gene Probe:**

Sequences of Probe: 5'-NCTTCATGAGTCAGTGCT-3'

Results:

Age Distribution Among Study Groups:

The patient's ages ranged from 19-72 years with a mean of 39.985 years. The mean age of patients with malignant ovarian tumors (42.58 years) was higher than the mean age of the borderline tumors & benign ovarian tumors (41.90 years) and (39.82 years), respectively. While, the mean age of apparently healthy control(AHC) was (35.64 years). However, there was highly significant differences at ($p<0.01$) between different groups in age distribution (Table 1).

Table (1): Distribution of ovarian Tumors patients according to their age.

Studied groups	N	Mean (Age / Year)	S.D	Std. Error	Range		ANOVA Test (P-value)
					Mini.	Maxi.	
Control	40	35.64	10.183	1.610	27	60	P=0.003 Highly sign. (P<0.01)
Benign tumor	45	39.82	7.646	1.140	19	55	
Border line tumor	20	41.90	10.126	3.202	29	57	
Ovarian cancer	45	42.58	8.083	1.205	25	72	
Total	150	39.985					

Histological Types of Malignant Ovarian Tumors:

Frequency distribution of histological types according to biological behavior was Serous epithelial (71.11%), Mucinous (20%) and Endometrium (8.89 %) (Figure1).

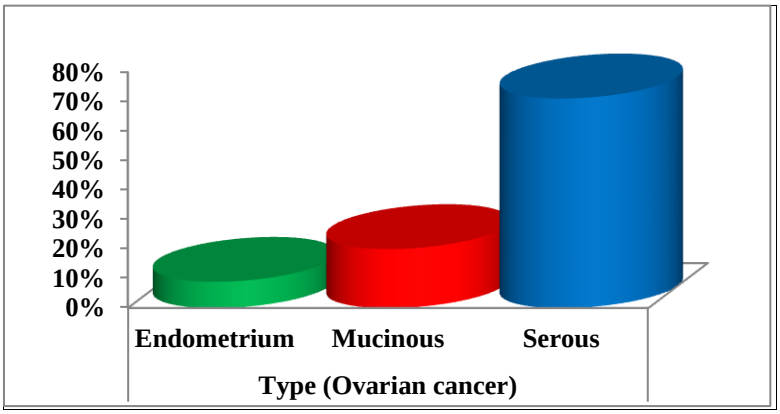


Figure (1): Distribution of malignant ovarian tumors group according to histological types.

Histopathlogical Grades of Malignant Ovarian Tumors:

The results of present study show that moderately grades ovarian carcinomas (grade II) constituted 35.5% (16 out of 45 tissues), whereas with poorly differentiated grade ovarian carcinomas (grade III) constituted 33.3% (15 out of 45 tissues) and well differentiated (grade I) 31.2% (14 out of 45tissues), respectively. The results reveal non-significant differences at (P>0.05) between poorly differentiated grade and well differentiated grade, also non-significant difference was noticed between poorly differentiated and moderately differentiated ovarian carcinomas (Table 2).

Table (2): Grading of ovarian cancers group.

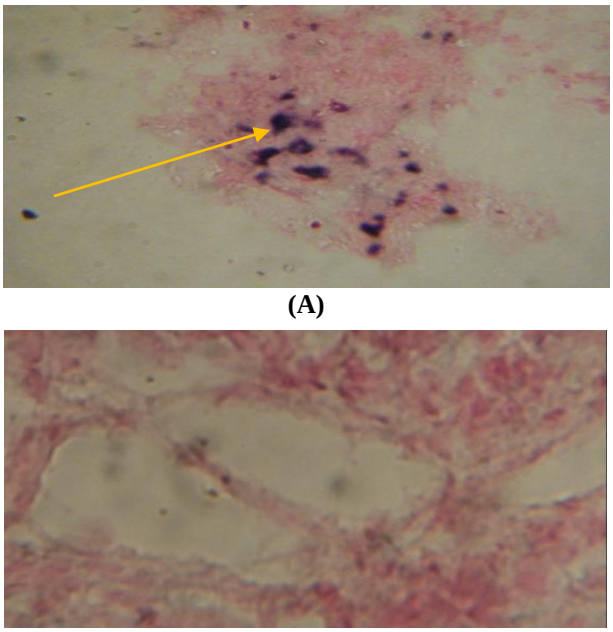
Ovarian Cancers		Total (N=45)	%	P-value
Grades	I	14	31.2	χ^2 test P=0.843 Non sign. (P>0.05)
	II	16	35.5	
	III	15	33.3	

Results of *Epstein Barr Virus* (*ZEBRA - EBV*) - Associated Ovarian Tumors:

The positive results of *ZEBRA - EBV* -CISH detection in malignant ovarian tumors, where 64.4% (29 out of 45 tissues) showed positive signals. While, in the benign ovarian tumors group was 37.8 % (17 out of 45 tissues), followed by borderline ovarian tumors & the apparently healthy ovarian control tissues were 30%(6 out of 20 cases) and 7.5% (3 out of 40 cases), respectively (Table 3).

Table (3): Distribution of *ZEBRA - EBV* DNA Signals with Ovarian Tumors.

ZEBRA – EBV		Studied groups				Pearson Chi-Square (P-value)
		A.H. Control	Benign tumor	Border line tumor	Ovarian cancer	
Positive	N	3	17	6	29	P=0.00 Highly sign. (P<0.01)
	%	7.5%	37.8%	30%	64.4%	
Negative	N	37	28	14	16	
	%	92.5%	62.2%	70%	35.6%	
Total	N	40	45	20	45	
	%	100%	100%	100%	100%	
Z test			P=0.222 NS	P=0.344 NS	P=0.072 NS	



(A)

(B)

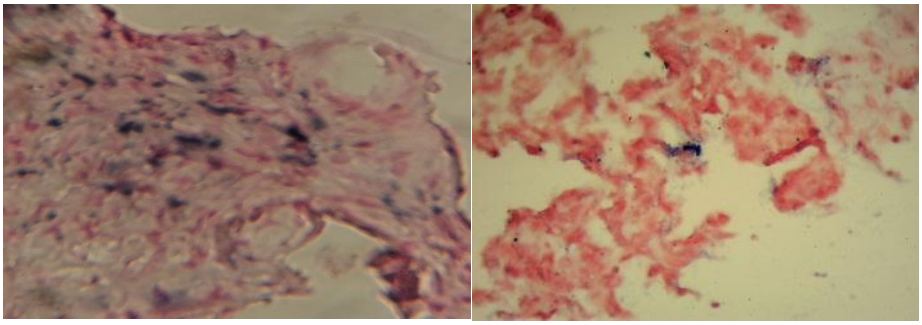
Figure (2): Microscopic appearance of *ZEBRA -EBV- CISH* signal in ovarian tumors. Using Digoxigenin-Labeled ZEBRA -EBV Probe; stained with 3-Amino 9-Ethyl Carbazole (Red) and Counter stained by Nuclear Blue Solution (Blue). Red signal are detected at complementarity sequences sites (arrows). A) *Positive ZEBRA -EBV* DNA, moderate score and strong signal intensity (X1000). B) Negative of *ZEBRA -EBV* DNA (X1000).

Results of *HCMV* in Female Patients with Ovarian Tumors:

(Table 4) shows the positive results of HCMV-CISH detection in malignant ovarian tumors, where 55.6% (25 out of 45 cases) showed positive signals, while, 44.4% negative signals, which represented 20 out of 45 cases in this group. While, in the benign ovarian tumor group was 35.6% (16 out of 45 cases). Negative signals which in benign group represented 29 out of 45 cases constituted 64.4% . Whereas ,the positive results in borderline ovarian tumor group was 40% (8 out of 20 tissues), followed by the apparently healthy ovarian control tissues was 12.5% (5 out of 40 tissues). Statistically, significant difference ($p<0.01$) was found on comparing the percentages of *HCMV* DNA among the study groups (Table 4).

Table (4): Frequency Distribution of Chromogenic *In Situ* Hybridization Signals for *HCMV* DNA among Study Groups

HCMV		Studied groups				Pearson Chi-Square (P-value)
		A.H. Control	Benign tumor	Border line tumor	Ovarian cancer	
Positive	N	5	16	8	25	P=0.003 Highly sign. (P<0.01)
	%	12.5%	35.6%	40%	55.6%	
Negative	N	35	29	12	20	
	%	87.5%	64.4%	60%	44.4%	
Total	N	40	45	20	45	
	%	100%	100%	100%	100%	
Z test			P=0.072 NS	P=0.754 NS	P=0.551 NS	



(A) **(B)**

Figure (3): Microscopic appearance of *HCMV- CISH* signal in ovarian tumors. Using Digoxigenin-Labeled *HCMV* Probe ; stained with 3-Amino 9-Ethyl Carbazole (Red) and Counter stained by Nuclear Blue Solution (Blue). Red signal are detected at complementarity sequences sites (arrows).A. Serous Ovarian Cancer, HCMV DNA, moderate score & strong intensity (X400). B.) Benign Ovarian Tumors , Positive *HCMV* reaction with low score and moderate signal intensity (400X).

Discussion:

Ovarian cancer is primarily a disease of postmenopausal women, with the large majority of cases occurring in women between 50 and 75 years old. The incidence of ovarian cancer increases with age and peaks at a rate of 61.5 per 100,000 women in the 75-79-year-old age group (12). On reviewing the 150 cases which were included in this study, it was found the age of the patients with ovarian tumors was ranging between 19-72 years and their mean age was 39.985 years (Table 1). The present results are consistent with those reported world-wide where these ovarian tumors were usually affecting females over forty years of age (American Cancer Society). Ovarian cancer is a disease of elderly women. It has been speculated that age can affect prognosis in ovarian cancer: an independent but yet unexplained association of advanced age with prognosis, possibly due to different tumor biology (13). Benign ovarian cysts are a common finding in women of all ages (14).

In 2004, in the United States, 50% of all ovarian carcinomas were bilateral. Malignant serous tumors constituted over 40% of invasive epithelial carcinomas. In the present study, was found the most common type the Serous epithelial (71.11%), followed by Mucinous (20%) and Endometrium (8.89 %) (Figure 1). These results are incompatible with several other studies such as (15) found that patients who have invasive serous carcinomas usually acquire more aggressive biological behavior of ovarian carcinoma. Endometriosis is a common gynecologic disorder. The estimated frequency among women of reproductive age is 5%–10% and is particularly frequent among women with pelvic pain and infertility. In 1925, Sampson was first to describe the malignant transformation of endometriosis to ovarian carcinoma (16).

The results of present study show that poorly differentiated constituted 35.5%, followed by moderately grades 33.3% and lastly, 31.2% for well differentiated (Table 2). These results gave us an indication the old age

women may be more susceptible to get malignancy for several reasons. Several factors related to these finding, the cell mediated immunity plays an important role in the defenses against the cancer. These results are compatible with Stanly report in 2005 who shows the importance of cellular immune responses in the resolution of viruses infection, it is not surprising that deficiencies in cell-mediated immunity increase the likelihood of disease expression (persistence or progression) in groups such as older women (waning immunity), transplant recipients, patients with HIV, and those receiving immunosuppressive drugs (12).

Studies on the prognostic implications of age and ovarian cancer are inconclusive. Chan and his colleague when reported that, the distribution of tumor grade differed between young and old women. They found that in younger women with mean age 40±5.7 years, well, moderately, and poorly differentiated carcinoma constituted 11%, 35%, and 54% respectively, compared to 4%, 11%, and 85% in older patients with a mean age of 61±8.7 (17). Similar findings were also reported by (18, 19), others have found that age is not an independent prognostic factor after adjusting for tumor stage and grade (20). While, by (15) who found high-grade clinically aggressive neoplasms that are usually diagnosed at an advanced stage.

Epstein Barr-Virus (EBV) establishes successful life-long persistence in healthy carriers. Because many EBV proteins have oncogenic potential and can be the targets recognized by the host immune system, the virus stays peacefully with the host by restraining viral replication and expression of latency program of viral proteins(21). However, aberrant reactivation of EBV from the latent phase into the replicative (lytic) phase is frequently linked to the development or progression of EBV-associated cancers(22). During primary infection, EBV initially undergoes a brief replication in the epithelial cells EBV infects human B lymphocytes and epithelial cells via different entry mechanisms. In contrast, the mechanism by which EBV infects human epithelial cells remains unclear(23). ZEBRA plays an indispensable role in driving the lytic cycle of EBV (24). EBV-encoded BZLF-1 protein (ZEBRA) downregulates NF-κB and promotes viral lytic growth and host cell apoptosis (17). Significantly high percentage of ZEBRA I&II detection in ovarian cancer group (64.4% for ZEBRA) was observed on comparison to border line , benign ovarian tumors and apparently ovarian control groups (Table 3). This finding reflects a possible role of the EBV-infection in the carcinogenesis of ovarian malignant tumors group, majority of these patients' tumors are EBV-positive. ZEBRA protein expressed in human T lymphocytes could alter T-cell proliferation and apoptosis during EBV infection (25), therefor results of tumor show in this study increased in malignant associated with EBV. These results are consistent to those reported by (9) Who elevated IgG titers to viral capsid antigen of *EBV* , a marker of a relatively severe (and, conceivably, later) initial EBV infection, had a 5.3-fold (95% CI 1.5-18.4) increased risk of ovarian cancer.

ZEBRA plays a fundamental role in disrupting latency and initiating the

EBV lytic cascade. Transcriptional activation of the ZEBRA-encoding BZLF1 gene is the primary underlying mechanism by which activators of lytic virus replication. ZEBRA shares homology with the DNA-binding domain of the cellular transcription factor (26). Because the lytic transactivator protein BZLF1 is necessary and sufficient to induce, the lytic cycle differences in this protein could help modulate the responsiveness to autoreactivation signals or to other inducers of the lytic cycle (27).

The reason for EBV to exert its oncogenic influences in a particular patient is unknown but is probably associated with co-factors. Again, it is possible that HPV exerts its oncogenic influences in concert with co-factors including a possible collaboration with EBV (28). The fact that we found viral DNA in healthy ovarian specimens could support, to a certain extent, the hypothesis that the virus might play a role in the etiology of ovarian cancer in only a subpopulation of patients. It is logical, on the other hand, to believe that the presence of EBV alone is not sufficient to implement the full carcinogenesis process and that further changes would accumulate over time in a stepwise manner to cause the disease and in turn suggesting a need for further large cohort studies to explore the role of each contributing factors.

Also, the differences of current results, might be related to the geographic variation, the sensitivity of the probe used for CISH, or differences between the subjects studied, yet a definitive reason is not apparent.

Up to our best knowledge, this is the first work in Iraq with a molecular design that used a recent sensitive version of CISH technique to demonstrate the DNA of the late gene of HCMV in Iraqi patients with different grades ovarian cancers. Chromogenic *In situ* hybridization methods for detection of nucleic acid sequences have proved powerful especially for revealing genetic markers and gene expression in a morphological context (29).

Here about, it has been chosen the molecular design so as to demonstrate the HCMV-DNA of the late gene (that encodes matrix protein of this virus) in Iraqi patients with ovarian cancer with different grades. In the current study, the HCMV DNA-CISH was detected in 55.6% malignant ovarian tumors. While, in the benign ovarian tumor group was 35.6% (16 out of 45) case Whereas ,the positive results in borderline ovarian tumor group was 40%, followed by the apparently healthy ovarian control tissues was 12.5% (5 out of 40 tissues) (Table 4).

Carlson *et al.* (10) was found 8/10 (80%), 4/9 (44%) and in 4/10 (40%), 5/8 in ovarian cancer tissue specimens have evidence of HCMV-IE and pp65 infection where their expression was based on CISH .This observation indicates that reactivation of latent HCMV within the tumor at IDS may be induced with NACT as both viral proteins could be detected in tumor tissue sections obtained from these 2 patients after treatment. Although unlikely, it can however not be excluded that the virus infected the tumor between the 2 test occasions. Furthermore, HCMV-β2.7 was detected at lower intensity in all examined tissue samples (n = 5) obtained at IDS after NACT. The observation that HCMV DNA and low grade HCMV-protein expression were detectable in tissue sections after NACT indicates that

HCMV may be present at low activity or in a latent phase in cells in these tissues. It is possible that latent HCMV could subsequently be reactivated under the influence of the inflammatory tumor microenvironment or by the chemotherapy per se. In our study, we could detect HCMV-IE and HCMV-pp65 proteins in the majority of tissue sections by carefully optimized IHC and HCMV-β2.7 DNA detection by ISH. This strengthens the previous report by (11), who examined the prevalence of HCMV in ovarian cancer tissues by polymerase chain reaction and found HCMV-glycoprotein DNA in 50% of the patients.

Inflammation is a key factor for the reactivation of latent HCMV. Previous studies have implied a potential role of inflammatory factors in the ovarian malignancy process (30). Active HCMV infection may aggravate the inflammatory microenvironment by increasing production of inflammatory factors such as IL-1β, IL-6, IL-8, and tumor necrosis factor-α, transforming growth factor-β, viral IL-10, prostaglandins, and leukotrienes (31). A number of proteins including both HCMV-pp65 and HCMV-β2.7 are important in providing immune evasion and antiapoptotic strategies. HCMV -pp65 is a major abundant viral tegument protein with enzymatic kinase activity that is involved in oncomodulation via immune evasion by downregulation of histocompatibility complex class (HLA)-I and II and preventing antigen presentation and immune system activity and preventing crosstalk between natural killer (NK) and dendritic (DC) cells by interfering with NKp30 activating receptor(32). Moreover, HCMV-pp65 contributes to immunosuppression by downregulation of the interferon response.

The virus ability to delay and prevent apoptosis may be as a consequence prevented the therapeutic action of chemotherapy in HCMV-infected tumor cells. Thus, the activity of HCMV in a tumor may promote disease progression and prevent desired effects of chemotherapy in some cancer patients (Rah Ovarian cancer patients have poor survival despite advances in molecular biology, diagnostic imaging, multidisciplinary surgical intervention, and oncological treatment (19).

Despite a limited number of specimens in this study, our findings may indicate a role for HCMV in ovarian cancer. Further studies should focus on validating these findings in a larger cohort of patients. Elucidating the potential role of HCMV in epithelial ovarian cancer is of great interest given the availability of antiviral therapies that may be active in this disease.

We concluded from this study, ZEBRA I -EBV genes as well as HCMV-DNA positive signals in malignant, borderline and benign tumors tissues, they suggest an important role for these viruses in the development of ovarian tumors in our Iraqi patients.

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