

**The effect of contraceptive pills, the menstrual cycle  
and the level of education on the incidence of breast  
cancer in Iraqi women**

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سرطان الثدي هو احد الامراض التي تصيب انسجة الثدي ينشأ من نمو غير طبيعي في قنوات أو فصيصات الثدي وهو أحد الأسباب الرئيسية للوفاة في جميع أنحاء العالم ، وهو السبب الأكثر شيوعاً للوفاة بين النساء ، وخاصة النساء الأمريكيات، وهو السبب الثاني للوفاة في العراق ، حيث يبلغ معدل الإصابة به من ١ إلى ٥ بالمائة ، وفقاً لمنظمة الصحة العالمية. هدفت الدراسة الى معرفة تأثير حبوب منع الحمل على مستوى تعبير HLA-G لدى المريضات المصابات بسرطان الثدي. كما هدفت الدراسة الى معرفة مدى تأثير الدورة الحوضية من ناحية انتظامها و عدم انتظامها على تعبير هذه الجزيئة المثبطة للمناعة، اضافة الى ذلك معرفة مدى تأثير المستوى التعليمي للمريضات على الإصابة بسرطان الثدي من خلال معرفة مستوى تعبير HLA-G. الطريقة: شملت الدراسة خمسين امرأة مصابة بسرطان الثدي ، بالإضافة إلى ٢٥ امرأة سليمة كمجموعة ضابطة. تم استخدام تقنية تفاعل البلمرة المتسلسل الكمي (qPCR) لقياس التعبير الجيني لجزيئة HLA-G. النتائج: أظهرت النتائج وجود فرقاً معنوياً في تعبير HLA-G لدى المريضات اللاتي تتناولن حبوب منع الحمل ( $5.197 \pm 0.819$ ) بيكوغرام / مل مقارنة بالمريضات اللاتي لم يتناولن حبوب منع الحمل ( $3.190 \pm 0.542$ ) بيكوغرام / مل ( $P \leq 0.01$ ). كما أظهرت النتائج وجود فرق معنوي في تعبير HLA-G بلغ ( $4.195 \pm 0.778$ ) بيكوغرام / مل في المريضات ذوات الدورة الحوضية المنتظمة مقارنة بالمجموعة الضابطة ( $1.127 \pm 0.128$ ) بيكوغرام / مل ( $P \leq 0.01$ ) ، وفرق معنوي في تعبيره لدى المريضات بعد انقطاع الطمث ( $3.549 \pm 0.697$ ) بيكوغرام / مل مقارنة بمجموعة التحكم ( $1.180 \pm 0.111$ ) بيكوغرام / مل ( $P \leq 0.05$ ) ، كما أظهرت النتائج الحالية اختلافاً معنوياً في تعبير HLA-G في المريضات ذوات المستوى التعليمي الأول والثاني ( $4.416 \pm 0.584$ ,  $4.218 \pm 0.287$ ) بيكوغرام / مل مقارنة بالمجموعة الضابطة ( $1.167 \pm 0.122$ ,  $1.072 \pm 0.110$ ) بيكوغرام/مل على التوالي ( $P \leq 0.05$  ,  $P \leq 0.001$ ). الاستنتاج من خلال النتائج الحالية ، نستنتج أن حبوب منع الحمل لها تأثير في زيادة التعبير عن HLA-G وبالتالي تقدم الورم وتطوره، كما أن لتطور الحياة وتغيير نمط المعيشة دور واضح في الإصابة بسرطان الثدي.

### Abstract

Breast cancer is one of the diseases that affect breast tissue that arises from abnormal growth in the ducts or lobules of the breast (Al-Abassi et al., 2018). Breast cancer is one of the leading causes of death worldwide and the most common cause of death among women (Ferlay et al., 2015). especially American women (Giaquinto et al., 2022), It is the second cause of death in Iraq, with an incidence of 1 to 5 percent, according to the World Health Organization (Kulhánová et al., 2017).

**The study aimed** to find out the effect of Contraceptive pills on the level of HLA-G expression in patients with breast cancer. The study also aimed to find out the effect of the menstrual cycle, In terms of regularity and irregularity, on the expression of this immunosuppressive molecule. In addition, knowing the effect of the educational level of the patients on the incidence of breast cancer through knowing the level of HLA-G expression.

**Method:** The study included fifty women with breast cancer,

In addition to the control group, which consisted of 25 healthy women. Quantitative polymerase chain reaction (qPCR) technology was used to measure HLA-G gene expression.

**Results:** The results of the gene expression of the HLA-G showed a significant difference in the patient with the intake of Contraceptive pills ( $5.197 \pm 0.819$ )pg/ml compared to patients that do not intake of Contraceptive pills ( $3.190 \pm 0.542$ )pg/ml ( $P \leq 0.01$ ). “Also showed that there was a significant” difference in the expression of HLA-G, which amounted to ( $4.195 \pm 0.778$ )pg/ml in patients with regular Pre-menopausal compared to the control group ( $1.127 \pm 0.128$ )pg/ml ( $P \leq 0.01$ ), And a significant difference in its expression in post-menopausal patients ( $3.549 \pm 0.697$ )pg/ml compared to the control group ( $1.180 \pm 0.111$ )pg/ml ( $P \leq 0.05$ ), The present results also showed a significant difference in HLA-G expression in patients at Education Level I and II ( $4.416 \pm 0.584$ ,  $4.218 \pm 0.287$ )pg/ml compared to the control group ( $1.167 \pm 0.122$ ,  $1.072 \pm 0.110$ )pg/ml respectively ( $P \leq 0.05$ ,  $P \leq 0.001$ ). **conclusion**

Through the current results, we conclude that birth control pills have an effect on increasing the expression of HLA-G and thus the progression and development of the tumor, and that the development of life and lifestyle changes have a clear role in the development of breast cancer.

Keywords: Breast Cancer, HLA-G, Contraceptive pills, Menstruation

## Aims of Study

**The study aimed** to find out the effect of Contraceptive pills on the level of HLA-G expression in patients with breast cancer. The study also aimed to find out the effect of the menstrual cycle, as well as its regularity or irregularity, on the expression of this immunosuppressive molecule. In addition, knowing the effect of the educational level of the patients on the incidence of breast cancer through knowing the level of HLA-G expression.

## Introduction

**Breast Cancer** is one of the leading causes of death worldwide in both developing and developed countries, and is the mostly common cause of death among women (Ferlay et al., 2015), especially American women (Giaquinto et al., 2022). Breast cancer arises from the ducts or lobules of the breast (Al-Abassi et al., 2018). Where one or both breasts become infected, the cells begin to grow out of control.

Breast cancer, as well as lung cancer, are among the most common types of cancer worldwide, with the total number of new cases diagnosed in 2020 representing 12.5% and 12.2%. Thus, 1 in 9 women will develop breast cancer at some point in their lives (Rehman et al., 2022), It is the second cause of death in Iraq, with an incidence of 1 to 5 percent, according to the World Health Organization (Kulhánová et al., 2017).

### Human leukocyte antigen-G (HLA-G)

The nonclassical human leukocyte antigen HLA-G are molecules that were first discovered at the maternal-fetal interface of EVT in the placenta (Rebmann et al., 2014; Alizadeh et al., 2016), in 1982, and in 1990 named HLA -G, there are seven different isoforms of HLA-G mRNA, and this diversity is due to the alternative splicing of seven exons. HLA-related isoforms G 2, G 3, and G 4; either HLA-G5, G 6, G 7 in soluble HLA-G (sHLA-G) isoforms; In addition to HLA-G1, it is found in both types of isoforms (Xu, Zhou and Wei, 2020).

Major histocompatibility complex (MHC) molecules bind peptides to present them at the cell surface to be recognized by immune cells receptor on natural killer (NK) cells, T lymphocyte, or mast cell. Such recognition plays a crucial part in autoimmunity, anti-bacterial, anti-viral and anti-tumor responses. HLA-G has a role in pregnancy, endometrial disease, endometriosis (Bai et al., 2020).

HLA-G plays a role in the mechanism of maternal and fetal immune tolerance, and HLA-G can be expressed on the surface of tumor cells

, infected sites and other pathologic microenvironments to confer tolerance. It also has a role in organ rejection, tumor migration, and autoimmune diseases (Ullah et al., 2019).

The increase of inflammatory cytokine, such as IL-1 $\beta$  and IFN- $\gamma$ , leads to stimulate the expression of HLA-G (Al-Mashhadani et al., 2022),

HLA-G inhibits CD4<sup>+</sup> T lymphocyte proliferation and induces CD8<sup>+</sup> T lymphocyte apoptosis in addition to preventing the cytotoxicity of natural killer cells (Moslehi et al., 2016; Olsson and Olsson, 2020).

Therefore HLA-G expression is associated with malignancy as well as associated with decreased survival rate (Ullah et al., 2019)

## Contraceptive pills

It is a type of birth control pill that is highly popular for its effectiveness and low side effects, in addition to its low cost, as it works to prevent the secretion of gonads through the negative reactions of estrogen and progesterin on gonadotropins, and also inhibits the ovaries and causes down regulation of the proliferation of endothelial cells uterus and increased apoptosis in women with endometriosis. Birth control pills affect the immune system which leads to a decrease in the number of macrophages and an increase in the number of natural killer cells and Tregs (Waiyaput et al., 2021).

There are three types of birth control pills: estrogen, progesterone, and progesterone combined.

Combined hormonal birth control pills estrogen and progesterone are the most common. Progesterone works to prevent pregnancy, while estrogen controls menstrual bleeding (Cooper and Mahdy, 2017).

Progesterone works to prevent pregnancy by preventing follicle growth and thus preventing ovulation, Progesterone also makes cervical mucus unsuitable and thus prevents sperm from penetrating the cervix (Cooper and Mahdy, 2017).

These pills can be used to treat some cases of menstrual disorders such as menstrual pain, irregularity and fibroids, as well as pain associated with endometriosis and migraines associated with the menstrual cycle (Maguire and Westhoff, 2011). It also reduces the risk of ovarian cancers by 27% and reduces the risk

of colon cancer by 18%. And that some preparations have an effect on the treatment of acne and hirsutism (Cooper and Mahdy, 2017).

However, the periodic hormonal stimulation of breast tissue by these pills is considered the most important hormonal factor in the pathogenesis and development of breast cancer (Olsson and Olsson, 2020).

### Aims of Study

This study aimed to evaluate the level of HLA-G expression as a biomarker of breast cancer pathogenesis in Iraqi women.

### Methods

**Subject:** Fifty blood samples were collected from Iraqi female patients newly diagnosed with breast cancer from the Medical City Hospital in Baghdad (Teaching Oncology) from April to September 2021. The average age of the patients was 48 years (30-65) years. The patients were diagnosed by the medical advisory staff. The patients were diagnosed by the medical staff using mammography and histological findings. It was found that the lesion was early in the patients and none of the patients had received chemotherapy, radiotherapy, or mastectomy before blood collection. In addition to, 25 blood samples were collected from uninfected women as a control group, with an average age of 49 years (30-67) years. RT-qPCR was used to find out the level of HLA-G gene expression relative amount of *HLA-G* was normalized against *GAPDH* (internal control). The primers were *HLA-G F* (5'-GAGGAGACACGGAACACCAAG-3') and *HLA-G R* (5'-GTCGCAGCCAATCATCCACT-3') for *GAPDH-F* (5'-TGAGAAGTATGACAACAGCC-3') and *GAPDH-R* (5'-TCCTTCCACGATACCAAAG-3') respectively used for qRT-PCR reactions. All primers were produced from (Alpha DNA Company, Canada). The total volume (20 µl) containing master mix (10 µl), Nucleas free water (6 µl), for each forward and reverse primer (1 µl), and cDNA template (2 µl). The condition of PCR was 94°C/30 minutes, followed by 35 cycles at 94°C/5 seconds for denaturation, then 15 seconds at the 60°C to annealing, and 20 seconds at 72°C.

The 2-ΔCt method was used to calculate the expression of HLA-G for both the patients and the control group (Livak and Schmittgen, 2001).

### Statistical Analysis

Statistical analysis was carried out using spss version 25 (Steel and Torrie, 1960).

### Results and Discussion

#### • Relationship of the fold of HLA-G expression with Contraceptive pills.

The results of HLA-G expression showed (table 1) that there was a non-significant difference, which amounted to (5.197±0.819)pg/ml in patients that intake of Contraceptive pills compared to the control group (1.493±0.095)pg/ml; on the contrary, it was found that there is a highly significant difference in its expression in patients that do not intake of Contraceptive pills (3.190±0.542)pg/ml compared to the control group (1.030±0.091)pg/ml (P≤ 0.001). Also the results showed that there was a significant difference in HLA-G expression in favor of patients with intake of Contraceptive pills (5.197±0.819)pg/ml compared to patients that do not intake of Contraceptive pills (3.190±0.542)pg/ml (P<0.01).

Table (1) Relationship of the fold of HLA-G expression with intake of Contraceptive pills in patient and control.

Contraceptive pill	FE of <i>HLA-G</i> gene						F-test	P-value
	Patients Group (No. 50)			Control Group (No. 25)				
	$2^{-\Delta Ct Pa.}/2^{-\Delta Ct Co.}$			$2^{-\Delta Ct Co.}/2^{-\Delta Ct Co.}$				
YES	26	52%	5.197±0.819	5	20%	1.493±0.095	1.955	0.06NS
NO	24	48%	3.190±0.542	20	80%	1.030±0.091	3.594	0.001***
F-test			4.033			5.867		
P-value			0.050**			0.024*		

Although the present results did not show a significant difference in the HLA-G expression in patients who took birth control pills compared to healthy subjects, the increase in its expression was clear and significant. Therefore, it cannot be neglected, and non-significance may be due to the small size of the sample

As for the current results, which showed a significant difference in HLA-G expression of patients who took birth control pills compared to patients who did not take these pills, it indicates the effect of these pills in increasing the HLA-G, expressed on the cancer cells surface in order to suppress immunity; Where the HLA-G molecule binds to special receptors on the surface of immune cells, such as T cells and natural killer cells, preventing their proliferation and leading to their death (Soroush *et al.*, 2016). Thus contributing to the development and progression of cancer. This study agreed with a study that confirmed the use of birth control pills. For more than 4 years, the risk increases by 1.52 times for developing breast cancer, and using it for more than five years increases the risk by 1.46 in women using birth control pills. (Soroush *et al.*, 2016). Studies have also shown a positive relationship between birth control pill use and the age at which breast cancer was first diagnosed, women who started using the pill at age 18 had the disease 4 years younger than women who started taking the pill at age 22-25 or older than age 30 (Imkampe and Bates, 2012).

• **Relationship of the fold of HLA-G expression with Menstruation.**

The results of HLA-G expression in table (٢) showed that there was a significant difference, which amounted to  $(4.19 \pm 0.778)$  pg/ml in patients with regular Pre-menopausal compared with control group  $(1.127 \pm 0.128)$  pg/ml ( $P \leq 0.01$ ); on the contrary, it was found that there is non significant difference in its expression in patients with irregular Pre-menopausal  $(5.853 \pm 1.230)$  pg/ml compared to the control group  $(0.845 \pm 0.281)$  pg/ml, the results showed

a significant difference in expression of HLA-G, which amounted to  $(3.549 \pm 0.697)$  pg/ml in patients with Post-menopausal compared with control group  $(1.180 \pm 0.111)$  pg/ml ( $P \leq 0.05$ )

The results showed that there was a non significant difference in the expression of HLA-G in Pre-menopausal and Post-menopausal patients.

Table (٢) Relationship of fold of HLA-G expression with Educational level in patient and control.

Menstruation		FE of HLA-G gene						F-test	P-value
		Patients Group (No. 50) $2^{-\Delta Ct Pa.} / 2^{-\Delta Ct Co.}$			Control Group (No. 25) $2^{-\Delta Ct Co.} / 2^{-\Delta Ct Co.}$				
		No.	%	Mean $\pm$ SE	No.	%	Mean $\pm$ SE		
Pre-menopausal	regular	28	56%	$4.19 \pm 0.778$	14	56%	$1.127 \pm 0.128$	2.762	009**
	irregular	7	14%	$5.853 \pm 1.230$	2	8%	$0.845 \pm 0.281$	2.071	077NS
Post-menopausal		15	30%	$3.549 \pm 0.697$	9	36%	$1.180 \pm 0.111$	2.599	.016*
F-test		.959			0.502				
P-value		.391NS			0.612NS				

The increase in HLA-G expression in women with regular menstruation compared to healthy subjects was consistent with the results of one study that confirmed that women with breast cancer were more likely to have regular cycles, short and more cycles than healthy women; Because the luteal phase is fixed in time, only the follicular phase may differ, so women who have shorter, more frequent cycle will experience higher amounts of progesterone during the luteal phase (Atashgaran *et al.*, 2016). Progesterone increases the risk of breast cancer although it protects against endometrial cancer (Brisken, 2013). During the luteal phase, greater numbers of dividing epithelial cells appear in the follicular phase (Huh *et al.*, 2016). And since cell division in general is a prerequisite for carcinogenesis, short, multiple cycles put women at increased risk of developing cancer due to increased cell proliferation. Shorter menstrual cycles have been associated with the cytochrome P450 17 (CYP 17) genotype (Olsson and Olsson, 2020).

Benign breast disease is distinguished by irregular menstruation, which often occurs near the end of childbearing age. Irregular cycles lead to cystic disease of the breast and ovaries, thus women with cystic

ovarian disease have a lower incidence of the breast cancer (Olsson and Olsson, 2020). The hormonal level plays an active role in the risk of breast cancer. The incidence was reduced by 45% in women who underwent oophorectomy before the age of 40 compared to women who underwent a natural menopause between the ages of 50 -54. In addition to, bilateral oophorectomy at an early of age has been related with a lower risk compared to natural menopause at the same age, as a consequence of the pronounced and abrupt decrease in endogenous hormones as a result of the surgery (Brinton *et al.*, 1988).

Therefore, the menstrual cycle and related hormone secretion has an important role in the emergence of cancer cells that are characterized by the expression of the HLA-G molecule in an attempt to suppress the immune response.

• **Relationship of the fold expression of HLA-G with Educational level.**

The results of table (3) are shown that there was a non-significant difference in the expression of HLA-G, which amounted to  $(0.342 \pm 0.000)$  pg/ml in patients how uneducated compared to the control group  $(1.428 \pm 0.342)$  pg/ml; on the contrary, it was found that there is a significant difference in its expression in patients at I and II level education  $(4.416 \pm 0.584, 4.218 \pm 0.287)$  pg/ml compared to the control group  $(1.167 \pm 0.122, 1.072 \pm 0.110)$  pg/ml respectively ( $P \leq 0.05, P \leq 0.001$ ). The results also showed that was a non significant difference in the expression of HLA-G in patients how uneducated compared to patients how educated.

Table (3) Relationship of fold of HLA-G expression with Educational level in patient and control.

Educational level	FE of HLA-G gene						F-test	P-value	
	Patients Group (No. 50)			Control Group (No. 25)					
	No.	%	Mean $\pm$ SE	No.	%	Mean $\pm$ SE			
uneducated	2	4%	$0.342 \pm 0.000$	2	8%	$1.428 \pm 0.342$	3.178	0.086NS	
Educated	I	43	86%	$4.416 \pm 0.584$	6	24%	$1.167 \pm 0.122$	2.060	0.045*
	II	5	10%	$4.218 \pm 0.287$	17	68%	$1.072 \pm 0.110$	12.461	0.000***
F--test			1.209	0.671					
P--value			0.308NS	0.522NS					

The reason for high level of HLA-G expression in the educated group may be attributed to the changing lifestyle at the present time

In view of the fact that most members of the educated class are employees, they do not find enough time for them to do some daily work, which leads them to eat fast food or foods that contain preservatives which are considered unhealthy diets. In addition to work requirements that may lead them to use smart devices such as computers, iPads, and mobiles for long hours, which exposes them to the risk of radiation emanating from these devices, in addition to delaying their supposed sleep times.

In addition, the psychological pressures resulting from work may contribute to the weakening of the immune system and thus the progression and development of breast cancer.

**conclusion**

Through the current results, we conclude that birth control pills have an effect on increasing the HLA-G expression and, thus the progression and development of the tumor, and that the development of life and lifestyle changes have a clear role in the development of breast cancer.

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