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Upregulation of Interleukin-6 in HPV-Positive Breast Cancer Patients

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ABSTRACT

Background: Interleukin-6 (IL-6) is a well-known proinflammatory cytokine with tumor promoting capacity in various forms of malignancies including breast cancer (BC). Data highlighted the substantial role of HPV in the pathogenesis of BC. Compelling evidence suggests the contribution of HPV in carcinogenesis through triggering inflammatory cytokines such as IL-6.

Objective: Here, we assessed the correlation between the presence of HPV infection and the status of IL-6 expression and serum level in BC.

Methods: 72 tissue specimens including tumoral (Case; n=36) and their adjacent normal tissues (Control; n=36) were used. Nested-PCR and Real-Time PCR were employed to identify HPV DNA and assess the expression of IL-6, respectively. In addition, 72 sera samples from BC patients (n=36) and an age-matched healthy control group (n=36) were taken to measure the IL-6 serum level by ELISA.

Results: Overall, the HPV DNA was detected in 19.4% (14/72) of samples. 33.33% (12/36) of cases and 5.5% (2/36) of the controls were found to be positive for HPV (P=0.003). The overexpression of IL-6 was observed in HPV+ samples compared to HPV- samples (P=0.05). However, the concentration of IL-6 serum level was remarkably different between patients and normal controls (P=0.0001. Intriguingly, IL-6 serum level was connected to the advanced clinical stage (III/IV), high grade (II/III), metastasis and, ER+ status of patients.

Conclusions: Our finding indicated that the overexpression of the IL-6 may be connected to HPV infection in BC. Furthermore, the results reinforced the clinical significance and prognostic value of the serum IL-6 in BC patients.

Keywords: Interleukin-6, Human papillomavirus, Inflammation, Breast neoplasm

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INTRODUCTION

Breast cancer (BC) is the most common malignancy among women and second leading cause of malignancy-related deaths worldwide (1). Several internal and external factors are involved in the pathogenesis of BC among which inflammation plays a remarkable role in the process (2). Inflammation has been considered as a hallmark of cancer and is found to be a key player in the progression and development of human malignancies (3). Chronic inflammation is able to initiate a variety of pathophysiological events to facilitate cellular malignant transformation and carcinogenesis through inflammatory cytokines such as the IL-6 (4).

The IL-6 is a leading pleiotropic pro-inflammatory cytokine that is produced by tumor cells and implicated in differentiation and proliferation of malignant cells that is known to be high in tumor tissue and serum of patients with diverse types of malignancies including prostate cancer, lung cancer, colorectal cancer, ovarian carcinoma and BC (5).The tumor-promoting features of the IL-6 originate from its ability in targeting all the characteristics of the tumorigenesis process including metabolism, apoptosis, survival, proliferation, angiogenesis, and metastasis (5). In cancerous tissue, the accumulation of pro-inflammatory cytokines at the cancer site participates directly in the development of the pro-tumorigenic microenvironment (6). Indeed, the IL-6 can take part in the formation of the cellular microenvironment which facilitates tumor growth (7). The elevation in circulating the IL-6 concentrations is widely believed to be relevant to breast cancer progression (8). The IL-6 can be produced in response to different stimuli such as viral infections. The upregulation of the IL-6 level has been fully documented in human patients chronically infected with viral agents (9). In this context, the role of oncogenic viruses in the exacerbation of the IL-6 has attracted tremendous attention as a result of their persistent nature.

Human oncogenic viruses are either causally connected or contribute to the development of human malignancies (10). Human papillomavirus (HPV) is a well□established cause of cervical cancer and is associated with various malignancies i.e., anogenital, head and neck(HNC), colorectal, and BC (11, 12). HPV belongs to the *papillomaviridae* family, a non-enveloped double-stranded DNA virus with a tropism to cutaneous and mucosal epithelial cells (13). Based on oncogenic properties, HPV is categorized into high-risk (HR-HPV) and low-risk types (14). Numerous investigators have highlighted a strong link between HPV infection and carcinogenesis of the breast (15-17). Most cases of HPV infections are asymptomatic and eliminated spontaneously by the immune system, however, persistent infection with HPV can result in the progression of malignant disease (18). Several lines of evidence have elucidated that persistent infection with HPV can trigger inflammation-mediated carcinogenesis (19-21). For this purpose, HPV has evolved strategies to serve inflammation-associated signaling pathways (22). These inflammatory signaling pathways provide critical crosstalk between inflammation and cancer, especially via their function in elevating the expression of cancer-promoting cytokines like the IL-6 (23). Previous studies have addressed the close relationship between HPV and dysregulation of the IL-6 to predispose the cells to tumorigenesis (24).

In the present study, we tried to elucidate the probable connection between gene expression and serum level of one of the most substantial cancer-promoting cytokines, the IL-6, and the presence of HPV infection in BC patients. Besides, we assessed the level of the IL-6 and the status of HPV infection according to clinicopathological parameters in patients with BC.

MATERIALS AND METHODS

Ethics Statement

All subjects who attended this investigation

signed informed consent. The research was performed under the Declaration of Helsinki and approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (Ethic number: IR.AJUMS.REC.1399.092).

Tissue and Serum Samples Collection

In this case-control study, seventy-two fresh frozen tissues comprising tumoral (n=36), and their adjacent non-tumoral tissues (ANTs, n=36) were obtained from 36 BC patients by fine needle biopsy from those patients referred to hospitals affiliated with Ahvaz Jundishapur University of Medical Sciences from April 2020 to October 2020. Newly diagnosed patients with confirmed histopathological findings were included and those participants who had a history of malignant disease and received preoperative adjuvant therapy such as chemotherapy and radiotherapy, who were pregnant, subjects with other severe organ diseases, inflammatory and autoimmune disorders such as rheumatoid arthritis were excluded. All pathological data were reviewed by an experienced pathologist and the TNM system was employed to classify various clinical stages of malignant breast tumors by consulting a well-experienced clinical group consisting of a surgeon, oncologist, and radiologist according to the World Health Organization (WHO) criteria (25). The percentage of the tumor within the tissue sections was estimated at 50%.

Tumoral tissues and ANTs were immediately snap-frozen in liquid nitrogen, transferred to the laboratory, and stored at -80°C for further assessments. Before tissue collection, blood specimens were drawn from all the enrolled subjects (n=36) and sera were taken and stored at -80 C for the serological tests. We also obtained 36 blood samples from age-matched healthy individuals with no history of malignancy, infections, and inflammatory disease for the control group. All the subjects completed a structured questionnaire comprising information about socio-demographics, lifestyle details, medical history, and other clinically relevant data.

Strict aseptic procedure is followed during the sample collection and handling of fresh tissue to avoid cross-contamination. For each sample, this standardized protocol contained the usage of separate disposable items including biopsy needles, and gloves and placing tissue biopsies individually in sterile cryotubes (sterile cryotube 2ml self-standing DNase/RNase free).

DNA Extraction and Quality Control

Tumoral and ANTs DNA extraction were prepared using QIAamp DNA Mini Kit) Cat No./ID: 51304 (according to the manufacturer's instructions. The quantity and quality of the extracted DNA were analyzed using NanoDrop 2000 (Thermo Scientific, USA). All specimens were subjected to β-globin amplification (110bp) to determine the DNA quality by using PCO3 /PC04 primers (Table 1) and positive samples underwent further analysis (Figure 1). All primers sequences used in this study are presented in Table 1.

Immense precautions were taken to prevent sample-to-sample cross-contamination in

Table 1. All primers sequences used in this study

Gene	Forward 5′- 3′	Reverse 5′- 3′	PCR size	Ta*
β-globin	ACACAACTGTGTTCACTAGC	CAACTTCATCCACGTTCACC	110 bp	55°C
HPV 1*	CGTCCMARRGGAWACTGATC	GCMCAGGGWCATAAYAATGG	450bp	55 °C
HPV 2*	TTTGTTACTGTGGTAGATACTAC	GAAAAATAAACTGTAAATCATATTC	150bp	42°C
IL-6	TGAACTCCTTCTCCACAAGCG	TCTGAAGAGGTGAGTGGCTGTC	151bp	59°C
GAPDH	GGTCGGAGTCAACGGATTTGG	TGATGACAAGCTTCCCGTTCT	140bp	59°C

*HPV1,2 represents first and second PCR and Ta shows temperature of annealing

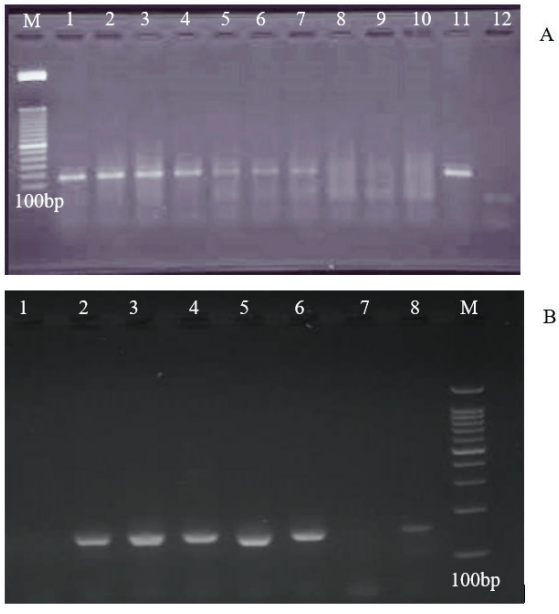

Figure 1. Agarose gel electrophoresis of PCR products. A: amplification of 110 bp fragment of β-globin: M: 100 bp DNA marker, lanes 1-7: positive samples, lanes 8-10: negative samples, lane 11; positive control, lane 12; Negative control. B: amplification of 150 bp fragment of HPV-L1 gene: Lane 1: negative sample, lanes 2-6: positive samples, lane 7: negative control, lane 8: positive control, M: 100 bp DNA marker

DNA extraction and PCR technique steps such as usage of a limited number of samples for processing per day, utilizing separate laminar flow cabinets (for extraction and PCRs) in physically separate rooms which were equipped with UV□light, usage of disposable RNase/DNase free filter tips and rigid DNA decontamination protocol. All pre-amplification and post-PCR steps were performed in different stations and negative and positive control were included in each PCR test.

Detection of HPV DNA and Genotyping

The nested-PCR was conducted to amplify the L1 region of HPV DNA using MY09/11 and GP5 + /6 + primer sets for the first and second round, respectively (Table 1). PCR reaction in a final volume of 25 µl contained 1X PCR buffer, 1.5 mM MgCl2, 100 µM dNTPs, 25 pmol of each primer (MY09/MY11) (GP5 + /GP6 +), 1 unit of Taq DNA polymerase, and 500 ng of DNA sample. The thermal conditions for the first and second round were as follows: pre-heat at 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 55 °C for 45s, and 72 °C for 1 min for amplification a 450 bp product and pre-heat at 95 °C for 5 min, then 35 cycles of 94 °C for 30 s, 42°C for 45s, and 72 °C for 1 min for amplification of a 150 bp product, respectively.

All positive samples were subjected to sequencing for HPV genotyping using ABI PRISM 3100 (Applied Biosystems) and 0.5 µL of appropriate internal primers. The comparative analysis for the obtained sequences was applied by nucleotide BLAST, BioEdit Package version 7.0.5.3, and Chromas software.

Gene Expression Analysis

The total RNA was isolated from tumoral and ANTs samples employing the Total RNA Minipreps kit (Bio Basic, Inc.) according to the kit instructions. RNA integrity was checked by 28S and 18S rRNA species preservation on agarose gel, and RNA yield was quantified via NanoDrop 2000 (Thermo Scientific, USA). cDNAs were generated by cDNA Synthesis Kit (Yekta Tajhiz Azma, YTA, Iran) from 2 µg of isolated RNA. Real-time PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA) with a specific primer sequence for the IL-6 and 2x Master Mix SYBR Green low ROX® (Amplicon, Denmark) (Table 1). For the IL-6 target, PCR reactions were carried out in a total volume of 25 µL containing 3 µg of cDNA 0.5mM each forward and reverse primers (Table 1). Thermal cycling conditions for the IL-6 included 15 min at 95 °C, 40 cycles of 15 s at 95 °C, 1 min at 59 °C. The relative expression level of the IL-6 was normalized utilizing the housekeeping gene GAPDH. All assays were performed in duplicate and relative quantification of the IL-6 was determined utilizing the $2^{-\Delta\Delta Ct}$ formula.

ELISA

The serum level of the IL-6 was measured

Table 2. Socio-demographic parameters and medical records of all subjects involved in study

Parameter		All cases	Case	Control	P value
Age	≤45	28	16	12	0.234
	>45	44	20	24	
Education	Primary school	34	18	16	0.878
	High school	28	13	15	
	Graduate	10	5	5	
Marital Status	Married	50	27	23	0.374
	Divorced	3	2	1	
	Single	19	7	12	
Children	Yes	44	23	21	0.629
	No	28	13	15	
No. Children	1-3	29	15	14	0.919
	>3	15	8	7	
Place of residence	Urban	56	26	30	0.257
	Rural	16	10	6	
Occupational status	Employed	20	9	11	0.284
	Unemployed	50	27	23	
	Unknown	2	0	2	
Family history of cancer	Yes	16	11	5	0.08
	No	56	25	31	
*History bacterial STDs	Yes	2	1	1	0.209
	No	67	32	35	
	Unknown	3	3	0	
History of viral STD	Yes	1	1	0	0.12
	No	68	32	36	
	Unknown	3	3	0	
Pap smear	Yes	26	15	11	0.49
	No	41	18	23	
	Unknown	5	3	2	
Pap smear	Normal	25	15	10	0.234
	Abnormal	1	0	1	
HRSB	Yes	1	1	0	0.12
	No	68	32	36	
	Unknown	3	3	0	
Menopause	Pre	38	19	19	0.193
	Post	31	14	17	
	Unknown	3	3	0	
Smoking	Non- Smoking	67	33	34	0.840
	Ex- Smoking	3	2	1	
	Current	2	1	1	
Alcohol consumption	Yes	3	2	1	0.555
	No	69	34	35	
Physical activity	Low	43	20	23	0.584
	Moderate	24	14	10	
	High	5	2	3	
Height	≤160	37	17	20	0.319
	>160	35	19	16	
Weight	≤60	41	17	24	0.07
	>60	31	19	12	
BMI	20-25	53	26	27	0.958
	25-30	15	8	7	
	>30	4	2	2	

Abbreviations: STDs; sexually transmitted diseases, HRSBs; high risk sexual behaviors;
*Two subjects had history of chlamydia trachomatis

by Human IL-6 ELISA® Kit (Cat No.ID/ KPG-HIL6 96; Karmania Pars Gene, Iran), Based on the manufacturer's instructions. The level of the determined cytokines is expressed in pg/ml.

Data Analysis

All the data were analyzed in IBM SPSS version 21.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 6 (La Jolla, CA, USA), then compared, utilizing the chi-square test. Mann-Whitney U test was used to compare quantitative characters. For normality of the IL-6 and ELISA data Kolmogorov-Smirnov test is used. The Spearman correlation coefficient was employed to test the correlations between serum and expression levels of the IL-6 according to HPV presence. Kruskal-Wallis test is employed to assess gene expressions and cytokines levels among the groups.

RESULT

Socio-demographic Status and Clinicopathological Characteristics

The median age of the patients and the healthy controls were 50.1±9.9 years and 50.1±1.6 years (range, 28–63 and 31-66 years), respectively. Table 2 displays lifestyle factors, socio-demographic status, and medical records including the history of viral and bacterial sexually transmitted diseases (STDs) and high-risk sexual behaviors (HRSB), for each volunteer. No significant difference was found among the patients and the controls regarding socio-demographic and clinically relevant parameters. The data showed that none of the subjects (in both groups) had previous viral sexually transmitted infections HBV, HIV, and HCV. Only one patient acknowledged a previous (treated) infection with genital herpes. In BC patients, 30 had invasive ductal carcinomas (IDC; 83.3%), and 6 had invasive lobular carcinomas (ILC; 16.6%). The available clinicopathological parameters (CPP) of the enrolled subjects are illustrated in Table 3.

The Detection of HPV in Tumoral Tissue and ANTs

Overall, HPV DNA was found among 19.4% of tissue samples (14 out of 72) (Figure 1). Molecular evidence displayed significantly higher HPV positivity in 33.33% (12 out of 36) of tumor samples as compared with 5.5% (2 out of 36) in adjacent normal tissue (P=0.003) (Table 4). The distribution of HPV genotypes consisted of HPV16 (n=8), HPV18 (n=2),

Table 3. Patient's clinicopathological parameters

Parameter		Number	%
Age	≤45	16	44.4
	>45	20	55.6
Tumor Type	IDC	30	83.3
	ILC	6	16.7
Histological Grade	Low (I)	10	27.8
	High (II/III)	26	72.2
Clinical Stage	Early (I/II)	25	69.4
	Advanced (III/IV)	11	30.6
Metastasis	Yes	9	25
	No	27	75
ER	+	23	63.9
	–	13	36.1
PR	+	15	41.7
	–	21	58.3
HER-2	+	19	52.8
	–	17	47.2

Table 4. Detection of HPV in tumoral and ANTs

HPV Type	Tumoral	ANTs	P value
16 (n=8)	8	0	0.003 **
18 (n=2)	2	0	0.151
31 (n=2)	1	1	1.000
6 (n=2)	1	1	1.000
Total (n=14)	12	2	0.003 **

HPV6 (n=1) and HPV31 (n=1) in cases and HPV6 (n=1) and HPV31 (n=1) in the control group. The analysis revealed that HPV16 was the dominant genotype present in the samples (57%, 8/14). In both the specimens in same person, neither infection of tumoral nor ANTs was observed (Table 4). There was no connection between the positivity of HPV infection and parameters such as the patient's socio-demographic status, medical history, and clinicopathological evidence as shown in Table 5.

The Status of IL-6 Gene Expression

Real-Time PCR (RT-PCR) demonstrated that the level of the IL-6 expression was significantly elevated among tumoral rather than ANTs (Figure 2; P=0.02). In HPV + tumor tissue, heightened IL-6 expression in comparison with HPV- tumor tissue and normal tissues was found (Figure 3A; P<0.05). Analysis pointed out non-significant

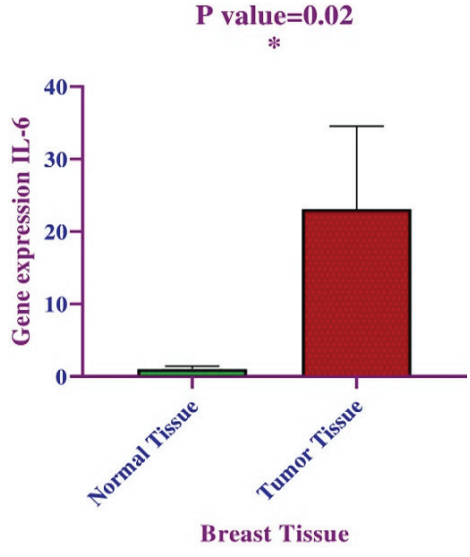

Figure 2. Mean expression level of IL-6 in tumor and ANTs.

results between HPV- tumor and normal tissues concerning the IL-6 expression (data not shown) (Figure 3A; P>0.05). Statistical analysis also demonstrated the differences in IL-6 levels between HPV negative normal

Table 5. HPV positivity, upregulated IL-6, and IL-6 serum concentration in breast cancer patients with respect to socio-demographic and clinicopathological features. Results are expressed as frequencies and mean±SEM.

Parameter		HPV	P value	Up IL6	P value	IL6 Con	P value
Age	≤45	6	0.635	21.9±16.9	0.249	23.1±1.8	0.838
	>45	6		21.7±14.3		25.6±3	
Tumor Type	IDC	10	1.000	25.5±12.8	0.394	25.3±2.1	0.467
	ILC	2		3.1±2.4		20.1±2.7	
Histological Grade	Low (I)	3	0.792	39.3±28.1	0.590	18.4±2.1	0.02*
	High (II/III)	9		15.1±10.5		26.8±2.3	
Clinical Stage	Early (I/II)	7	0.306	13.2±10.6	0.195	21±1.1	0.04*
	Advanced (III/IV)	5		41.4±25.8		32.4±4.9	
Metastasis	Yes	3	1.000	11.6±8.6	0.830	36.2±5.1	0.004**
	No	9		25.2±14.1		20.5±1.1	
ER	+	6	0.220	29.1±16.6	0.897	28.2±2.4	0.005**
	_	6		8.9±5.3		17.9±1.9	
PR	+	5	1.000	9.8±6.4	0.409	24.7±3	0.657
	_	7		30.3±17.9		24.3±2.4	

HER-2	+	7	0.637	16.1±13.9	0.510	26.2±2.4	0.257
	-	5		28.1±17		22.5±2.9	
Education	Primary school	7	0.709	6.3±3.8	0.856	22.4±2.9	0.354
	High school	4		31.1±22.04		26.8±2.8	
	Graduate	1		53.5±53.2		25.6±3.9	
Marital Status	Married	8	0.229	25.03±14.2	0.183	24.8±1.9	0.625
	Divorced	0		0.6±0.05		20.9±0.18	
	Single	4		15.5±9.1		24.4±6.7	
Children	Yes	9	0.326	13.7±12.3	0.08	24.2±2.3	0.845
	No	3		36.09±20.5		25±3.3	
No. Children	1-3	6	0.907	36.09±20.5	0.975	25±3.3	0.681
	>3	3		1.9±1.1		22.7±2.5	
Place of residence	Urban	7	0.188	25.6±14.7	0.903	21.4±1.05	0.07
	Rural	5		11.9±7.7		32.5±5.6	
Occupational status	Employed	4	0.414	7.6±7.2	0.486	24.6±4.8	0.869
	Unemployed	8		26.5±14.1		24.4±1.9	
	Unknown	-		-		-	
Family history of cancer	Yes	3	0.609	33.06±25.9	0.435	19.2±2	0.054
	No	9		16.9±10.9		26.8±2.4	
History of bacterial STDs	Yes	1	0.357	-	0.198	-	0.645
	No	10		13.2±8.4		24.4±2.1	
	Unknown	1		25.9±25.5		26.4±2.9	
History of viral STDs	Yes	0	0.779	-	0.339	-	0.374
	No	11		22.1±12		24.5±2.1	
	Unknown	1		25.9±25.5		26.4±2.9	
Pap smear	Yes	6	0.76	1.8±1.1	0.069	25.4±3.1	0.686
	No	5		26.2±15.1		24.8±2.5	
	Unknown	1		95.3±95		17.8±5.6	
Pap smear	Normal	6	NA	1.8±1.1	NA	25.4±3.1	NA
	Abnormal	0		-		-	
HRSBs	Yes	0	0.773	-	0.339	-	0.374
	No	11		22.1±12		24.5±2.1	
	Unknown	1		25.9±25.5		26.4±2.9	
Menopause	Pre-	7	0.883	17.2±13.9	0.929	23.2±19	0.616
	Post-	4		27.1±20.4		25.8±4	
	Unknown	1		25.9±25.5		26.4±2.9	
Smoking	Non- Smoking	12	0.441	22.8±11.7	0.997	24.5±2	0.739
	Ex- Smoking	0		15.55±15.53		23±0.4	
	Current	0		-		-	
Alcohol consumption	Yes	1	0.607	7.8±7.7	0.762	13.5±6.8	0.051
	No	11		22.6±11.4		25.1±1.9	
Physical activity	Low	6	0.825	18±13.6	0.996	21.3±1.1	0.075
	Moderate	5		30.3±20.2		27.6±4.2	
	High	1		0.55		34.3±7.2	
Height	≤160	6	0.813	6.9±4	0.07	24.5±3.6	0.471
	>160	6		35.1±19.9		24.4±1.5	
Weight	≤64	7	0.345	18.4±16.7	0.121	24±2.9	0.531
	>64	5		24.8±14.4		24.9±2.4	
BMI	20-25	8	0.823	13.4±10.9	0.08	24±1.9	0.421
	25-30	3		54.5±32.2		27.4±5.7	
	>30	1		0.55		18.3±2.7	

Abbreviations: Up IL-6: upregulated IL-6, IL-6 Con: IL-6 concentration ER: estrogen receptor, PR: progesterone receptor.

* Statistically significant result

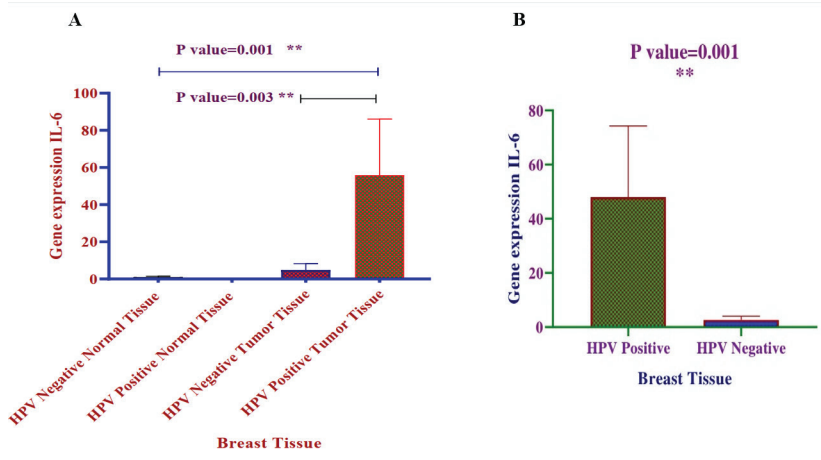

Figure 3. A: Comparison of IL-6 expression within tumoral and normal categories in reference to HPV status B: IL-6 gene expression with respect to HPV presence

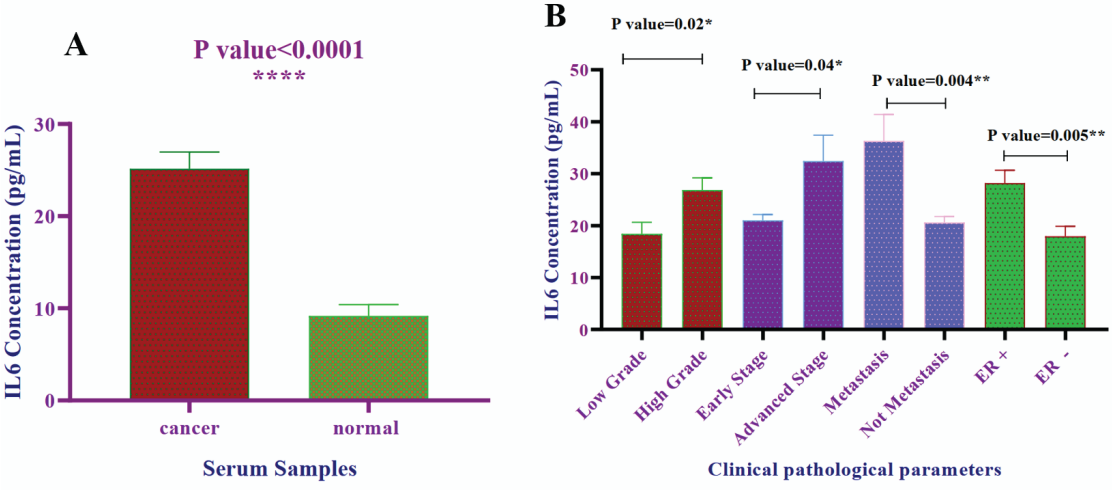

Figure 4. A: IL-6 serum concentration in patients and normal subjects B: Comparison of IL-6 serum level based on grade, stage, metastasis and ER status. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001

tissues and HPV positive normal tissues were not statistically significant (data not shown) (Figure 3A; P>0.05).

According to HPV infection status, the expression levels of the IL-6 were found to be meaningfully higher in HPV-positive compared to HPV-negative samples (Figure 3B; P=0.001).

The Measurement of Circulating IL-6 Concentration

72 Sera specimens from 36 patients with BC and 36 age-matched healthy control were subjected to determination of the IL-6 concentration by ELISA. Dramatic elevation of the IL-6 level in BC patients (24.5±1.8pg/ml) as compared to the healthy volunteers (9.1±1.2pg/ml) was observed (Figure 4A; P=0.0001) (Table 6). Interestingly, there was a statistical difference between advanced clinical stage (III/IV), high histological grade (II/III), and expression of ER and increased IL-6 serum concentration (Table 5; All P-values were less than 0.05) (Figure 4B). We noticed that the IL-6 concentration was significantly raised in metastasized patients (Figure 4B).

Median serum concentrations of the IL-6 within the patients did not display a considerable result concerning the presence of viral infection and the IL-6 expression as well (Table 6). The mean of circulating IL-6 concentration in HPV + and HPV- group were 23.5±3.6 vs. 25.1±2.1 pg/ml, respectively (P>0.05).

Table 6. Circulating IL-6 concentrations in BC patients(case) and healthy volunteers (controls), HPV+ and HPV-, and up- and downregulated IL-6 groups.

	Mean±SEM	P value
Case n=36	24.5±1.8	0.0001****
Control n=36	9.1±1.2	
HPV + n=14	23.5±3.6	0.089
HPV – n=22	25.1±2.1	
Up IL-6 n=16	26.7±2.6	0.095
Down IL-6 n=20	22.7±2.6	

* Statistically significant result

The serum level of the IL-6 did not differ remarkably concerning the expression of the IL6 (Table 6; P>0.05).

DISCUSSION

Current knowledge represents a direct causal interplay between chronic inflammation and carcinogenesis (26). Inflammation can stimulate tumor formation via two mechanisms, it first impairs the immune system to inhibit anti-tumor response, and second, it mediates cell proliferation and instability in the genome, resulting in oncogenic mutations (6). Numerous investigations have introduced the IL-6 as a leading well-characterized pro-tumorigenic marker in the development of inflammation-associated cancers. Upregulation of the IL-6 is extensively utilized by malignant cells such as epithelial oncogenic cells in favor of tumorigenesis (27). The IL-6 is produced in response to different stimuli such as viral infections. Human tumor viruses-mediated IL-6 upregulation can be achieved as a result of inflammatory signaling cascade activation (28). HPV-mediated cancers are believed to be a growing global health issue in upcoming decades (29). HPV infections account for approximately 5% of all malignancies worldwide (30). It is accepted as a fact that HPV-induced chronic inflammation can strongly promote neoplastic transformations in human cells (19). Evidence suggests the impact of HPV infection on the IL-6 gene expression, serum level, and its downstream clinical consequences (19). However, the exact relation in the context of breast carcinogenesis has not been researched.

In the current investigation, we initially assessed the presence of HPV DNA in the tumoral and adjacent normal tissues. Totally, 19.4% of samples were positive for HPV. Independent data have detected varying prevalence rates of HPV DNA ranging from 0 to 86% in tissues of BC (31). HPV prevalence can vary by geographic region, method sensitivity, and tissue type. In breast tissue, comparative analysis between fresh and paraffin-embedded tissues indicates HPV prevalence is higher when HPV DNA was isolated from fresh tissues than when HPV DNA was isolated from paraffin-embedded tissues (32). A performed investigation in Iran on paraffin-embedded BC tissues detected no HPV in any of the malignant and the normal control groups (33). Herein, we sought the molecular presence of HPV in the tumoral rather than corresponding fresh ANTs. HPV frequency was significantly higher in the tumoral rather than ANTs (33.33% vs 5.5%; P=0.003) (Table 4). In accordance with our research, a number of investigations have

identified a higher rate of HPV in malignant than normal cases (34-36).To our knowledge, there are a few works that utilized adjacent normal tissue of BC (37). Within the present study the distribution of HPV types were as follows: 16(n=8), 18(n=2), 31(n=1) and 6(n=1) in case and 31(n=1) and 6(n=1) in the control (Table 4). In our patients, all these detected genotypes are proposed to be involved in the pathogenesis of BC across the different populations worldwide (38). HPV 16 is considered the dominant type by previous studies (34). HPV 16 is also proposed to be among the most commonly detected types with the highest ability to develop cancer (39). In this study, HPV16 is the most prevalent type only found in the case group (Table4).

Among the tumor viruses, HPV is connected to the dysregulation of the IL-6 and its associated signaling pathways in cancer cells (40). On the other hand, evidence displayed BC cells produce an increased level of the IL-6 which can lead to proliferation, angiogenesis, epithelial-mesenchymal phenotype, and metastasis (41). Based on this hypothesis, we attempted to determine the gene expression level of the IL-6 in the tumoral and ANTs and among HPV+ and HPV- samples. In our study, RT-PCR showed the higher expression of the IL-6 in the tumoral vs. normal tissue (Figure 2; P=0.02). A meaningful increase of the IL-6 in HPV+ tumor samples in comparison to HPV- tumors and normal controls were found (Figure 3A; $P<0.05$). In this line, the difference between HPV- tumor and normal tissues was not significant (data not shown) (Figure 3A; $P>0.05$).

Consistent with the present result, an investigation explored the expression levels of inflammatory markers including the IL-6 in HPV-related BC. Researchers uncovered a statistically significant IL-6 elevation in BC tissues infected with HPV (42). They proposed HPV-elevated inflammatory cytokines such as the IL-6 could be considered as a prerequisite for BC progression. A distinctive aspect of our study vs. previous reports is related to finding the differences in the two groups tumoral and ANTs based on HPV positivity. According to HPV positivity, our analysis considerably indicated the enhanced expression of the IL-6 in HPV+ compared to HPV- patients (Figure 3B; P=0.001).

In other HPV-related malignancies, observations represent viral presence corresponds with the overexpression of the IL-6. In 2020, Moghoofei et al. performed a case-control study to test the potential contribution of HPV in lung cancer pathogenesis through inducing inflammatory markers including the IL-6 (43). This study indicated an increase in expression of inflammatory factor IL-6 in HPV positive specimens rather than HPV negative specimens. The authors have mentioned the probable effect of HPV on microenvironmental alterations by means of pro-inflammatory cytokines secretion, suggesting the promotion of epithelial proliferation.

An experimental study by Irigaray et al. described the overexpression of the IL-6 as attributed to HR-HPV oncoproteins within the infected cells which can largely promote pro-inflammatory/pro-liferative microenvironment and results in oncogenesis (44). Importantly, the major proportion of our HPV genotypes which was detected in breast cancer patients belonged to the HR-HPV category (12/14, 85%) (Table 4). In the context of HPV-associated malignancies, two recently published pieces of literature by Khodabandehlou (Iran, 2019) and Nahand (Iran, 2020) have exhibited the higher IL-6 expression in HPV + cases than HPV – cases (45, 46). In accordance with our result, these investigations have suggested that the major part of genotypes distribution is related to HR-HPVs, proposing the considerable role of HR-HPV oncoproteins in this event.

A body of works has illuminated that HPV employs tumorigenic inflammatory pathways, including nuclear factor-kappa B (NF-κB) and signal transducer and activator of transcription (STAT) to drive IL-6 (24, 47). Interestingly, produced IL-6, in turn,

is capable of affecting these mentioned oncogenic pathways to initiate a positive reciprocal interplay, sensitizing the cells to malignant phenotype (24, 44, 48). In other words, the putative role of the IL-6 in inflammatory loops may be considered as a major mechanism by which cancerous cells achieve aggressive phenotypes. For example, the IL-6/STAT3 axis is a well-known signaling pathway involved in a range of tumorigenesis features including proliferation and migration that has been activated in many malignancies including BC (49). Intriguingly, Ren et.al introduced highjacking of the IL-6/STAT3 signaling in cervical epithelial cells infected with HPV (47). They presented strong data that HR-HPV oncoproteins activate STAT3 by which mediates upregulation of the IL-6 and its secretion into the cellular matrix. Authors further showed that the IL-6 heightened activation of STAT3 in an autocrine manner, suggesting the remodeling of the microenvironment to favor the development of cervical cancer. Collectively, these data reinforce the notion that HPV may provoke a series of synergic events to enhance the expression of carcinogenesis-promoting genes in the process of oncogenesis.

In the course of HPV-induced inflammation, it should be noted that inflammation, in turn, can enhance HPV oncogenicity. Integration of HPV DNA into the cellular chromatin is a substantial step in the pathogenesis of HPV-associated cancers. Indeed, inflammation generates reactive oxygen species that stimulate DNA strand breaks, as a key prerequisite for HPV integration and consequently facilitating malignant transformation (50). Therefore, upregulation of inflammatory cytokines may induce a positive feedback loop in favor of HPV-related breast carcinogenesis, however, further studies should be performed.

Increase in expression of pro-tumorigenic cytokine IL-6 as a strategy to drive inflammation-mediated tumorigenesis is not limited to HPV and can be served by other viruses (51). Epstein-Barr virus (EBV) is a member of herpesviruses that are recognized as a class I carcinogen and accounts for a series of human cancers with the origin of lymphoid and epithelial (28). EBV along with HPV are the two most highlighted DNA oncoviruses in the pathogenesis of BC. A recently published work by Mostafaei et al. confirmed a relationship between the status of EBV tissue positivity and upregulation of inflammatory genes like the IL-6 in the context of breast carcinogenesis (52). They indicated higher expression of the IL-6 in EBV-positive tissue as compared with EBV-negative tissue**.** In another report by Mostafaei et al. in 2018, the increased level of cellular inflammation-related genes expression in EBV-associated thyroid cancer was reflected (53). The authors described significant elevation in the IL-6 and NF-κB levels in EBV□positive cases versus EBV-negative cases and introduced the probable participation of viral expressed proteins in the process of thyroid tumor promotion. In the current research, the presence of viral infection and cytokine expression were assessed regarding the socio-demographic and medical relevant parameters (Table 5). Analysis suggests these parameters may not affect the status of HPV infection and the IL-6 expression which is similar to previous investigations (54).

In order to better understand the clinical importance of inflammatory markers in persistent human papillomavirus infection and based on the emerging evidence illustrating the link between the heightened level of circulating the IL-6 and aggressive tumor progression and lower survival of various malignancies such as BC (55), we tested all sera taken from BC patients (n=36) and the healthy control group (n=36) for measurement of circulating cytokines by ELISA.

Our present result identified that the patient's IL-6 serum level was remarkably elevated in comparison with the healthy control group (24.5±1.8vs. 9.1±1.2 pg/ml) (Figure 4A; P=0.0001) (Table 6). Regarding clinicopathological characteristics, there was

a statistical association between the advanced clinical stage(III/IV), high histological grade(II/III), and ER+ condition with an increase in IL-6 serum concentration in BC patients (Table 5; $P<0.05$) (Figure 4B).

In agreement with our results, accumulating data suggest elevated IL-6 serum level is a crucial player in systemic inflammation that is usually accompanied by poor prognosis as a result of its link with advanced stages and high grades of tumors in the context of breast carcinogenesis (56-58). Moreover, we noticed that the IL-6 concentration wassignificantly raised in metastasized patients (Figure 2E, Table 5). Given that, our data may support previous findings introducing the IL-6 as one of the most characterized tumorigenic cytokines involved in breast cancer metastasis (58, 59).

The present study failed to confirm a considerable finding in the IL-6 serum concentration status according to HPV positivity and the IL-6 gene expression (Table 6; $P>0.05$). Relying on the type of HPV-related cancer, there are several lines of controversial reports on the interactions between the circulating level of the IL-6 and the status of HPV infection. For example, in a prospective study on cervical cancer, the level of circulating inflammatory marker IL-6 was significantly increased in HPV+ cancer rather than in the control sera (60). In contrast, multiple studies in HPV + HNC patients and HPV- HNC have mentioned either upper level of circulating IL-6 in HPV- HNC or no remarkable difference between the two groups (61). To our knowledge, we are the first study reporting serum level of the IL-6 considering HPV infection in BC patients. We examined the serum level of the IL-6, as a systematic evaluation of pro-inflammatory cytokines in the context of viral infections-correlated breast carcinogenesis.

CONCLUSION

The current investigation may provide several lines of evidence in the context of HPV-related BC through affecting inflammatory cytokines. We noted that the patients which were infected by HPV had a higher expression of the IL-6 rather than those who were not infected. We also displayed a strong difference in the concentration of the IL-6 serum level among patients and the healthy subjects. There was a substantial relationship between the increased level of circulating IL-6 and adverse clinical consequences (high grade and advanced stage) of BC patients, proposing consideration of the IL-6 serum level as a useful biomarker for assessment of malignancy prognosis. With respect to the presence of HPV infection, we failed to find a significant result in the patient's IL-6 serum level. However, a large body of "in vivo" and "in vitro" work appears to be necessary.

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