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Herpetic Oncogenic Virus Co – Infections Impinge Cell Cycle Regulatory Gene Expressions in Oral Cavity Malignancies

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ABSTRACT
Oral squamous cell carcinoma (OSCC) is the most encountered type of oral malignant neoplasm. P27 is a nuclear cell cycle inhibitory protein, that can be transported to the cytoplasm to deactivate it's role, Similarly the mechanism is used by cancer cells to unlock cell cycle arrest. In reaction to damage to DNA caused, either by the cell cycle arresting or apoptosis, the tumor suppressor p73 is activated. Epstein-Barr virus (EBV) that infects human beings has oncogenic capacity in different kinds of human neoplasia. Human Cytomegalovirus (HCMV) is a prevalent opportunistic pathogen that causes severe disease in various immunosuppressed patient. The aim of this study is to investigate the impact of EBV and HCMV contagions togetherness on p27 and p73 gene products in Oral cavity carcinomas. This study included a total number of seventy three (73) formalin-fixed, paraffin-embedded tissue blocks from OSCC patients. The confirmatory re-examination for histopathological samples were done; (a Chromogenic In Situ Hybridization assay (CISH) for EBV-EBERs and HCMV-PP65 DNA localization and immunohistochemistry assay (IHC) for p73 and p27 gene expression were performed). The results illustrate that the presence of these two viruses in OSCC were at highest percentage in undifferentiated grade (74.47%%). On the other hand, co-presence of p27 and p73 was highest in undifferentiated group (70.27%).
Keywords: OSCC, EBV-EBERS, HCMV-PP65, CISH, IHC.
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INTRODUCTION

The sixth greatest common malignancy in the world is oral cancer, the common oral carcinoma is the oral squamous cell carcinoma (OSCC); (It consists of over 90% of the total oral cavity malignant tumors) (1,2). The population age group <40 years was most affected (3). The global oral cancer prevalence was more than 300,000 cases in a year. Oral cancer mortality rate (approximately 2 per 100,000) in the Middle East as stated by World Health Organization (WHO) and that lower than that reported in India and the United States; on the other hand, there were 1664 OSCC cases in Iraq from (2001-2013) (4,5). The origin of OSCC from (non-keratinizing stratified mucosal epithelium); the significant environmental risk factors for OSCC development are (alcohol consumption ,tobacco and betel quid) (6); further causes may be occur to help cells overcome physiological proliferation control, such as; genetical predisposition, diet and oncogenic viruses. Virus of Epstein Bar (EBV) and human cytomegalovirus (HCMV) are the most the viruses associated with oral cancers (7,8). Keisuke et al., (1995) and González-Moles et al., (1998) concluded that EBV infection can be carcinogenic with oral squamous epithelium or, (the virus could only be found in oral squamous cell carcinoma epithelial cells instead.) (9,10). As well as, has been proved the ability of HCMV to control host gene expression, oncomodulatory, may be oncogenic function(11). The HCMV infection might building up the certain type of tumor cells via protection of it from apoptosis and modulating angiogenesis(12).

The p27/kip1 (p27) tumor suppressor inhibits cyclin/cyclin-dependent kinase (CDK) complexes and stops cell cycle progression. The p27 suggesting as an oncoprotein functions via further regulation of invasion and migration in cancer cells, (13).The p73 gene (a family member of p53 was first discovered in 1997), and this gene at that time has been studied extensively in cancer biology (14). The P73 have two main isoforms classes; (TAp73 acts as a tumor suppressor, whereas in contrast ΔNp73 act in a dominant negative mode as an oncogene which opposes the functions of TAp73 and p53) (15). The silencing or deletion of p73 gene proposing it's a possible role as tumor suppressor gene in (pancreatic cancers, neuroblastoma, breast and hepatocellular carcinomas) (16,17).

MATERIALS AND METHODS

This analysis enrolled a total number of seventy three (73),selected formalin-fixed, paraffin-embedded blocks from oral squamous cell carcinoma tissues; (these collected from histopathological laboratories archives of several hospitals in Baghdad). These blocks were belong to the previous 4 years (2016, 2017, 2018 and 2019); (are including 55 male, 18 female). The accompanied pathological reports of the corresponding patients were depended on it for diagnoses and the histopathological sections of biopsies stained by hematoxylin and eosin for final definitive diagnosis.

The Chromogenic in situ hybridization (CISH) reactions kit were used for detection of EBV and HCMV (obtained from ZytoVision GmbH. Fischkai, Bremerhaven. Germany) (Cat. Numbers:T-1061-40); the reaction conducted on 5µm tissue sections embedded in paraffin and (digoxigenin-labeled oligo-nucleotides probes were used which goals EBV-EBERs and HCMV-pp65 DNA (Cat. Numbers: T-1114-400 and T-1113-400).

The comprehensive methods for performing CISH reactions were performed according manufacturing company instructions. The evaluation of p73 and p27 protein expressions in OSCC tissues by using Immunohistochemical (IHC) test; the Monoclonal Rabbit Anti-P73 and Anti-p72 antibodies (Cat. Numbers: ab40658 and ab32034) were used and rabbit specific HRP/DAB detection IHC Kit, Abcam /England) that targeted nuclear specific proteins. The CISH and IHC signals counting were assessed at power (100X)

under light microscopy, (the scoring and intensity of signals were done according to (18)).

STATISTICAL ANALYSIS

The (version-21) SPSS program was used in this study for statistical analysis; where (Chi-Square test ($\chi 2$) was used to assess the significances between variables; and Spearman's rho statistical testing to evaluate studied markers scoring and intensities).

RESULTS AND DISCUSSION

Co-infection of CMV and EBV with Malignant Tumors:

Table (1) illustrates the correlation of co-infection of EBV and CMV in association with the grading of Oral Cavity Carcinoma (OCC). The undifferentiated grade have highest co-infections were found (74.47%) followed by well differentiated grade (14.89%) then poor differentiated grade (6.38%) and lastly by moderate differentiated grade (4.26 %). On the other hand, the percentage of HCMV- DNA alone in undifferentiated and moderately differentiated tissues were (85.71% and 14.29%, respectively) while none was found in other differentiated groups for each. The EBV- EBERS levels alone were (23.08% and 69.23 % in well and undifferentiated tissues, respectively). Statistically, these results showed non-significant differences among them according to grading system (P>0.05).

What is worthy to be noticed in the current study is that the co-infection of EBV and HCMV was recorded repeatedly (80.85%) in oral cavity (OC) malignant tissues with advance stages (as in Table 1).

Saleem et al., (2019) who found a significant statistical relationship of EBV infection with oral squamous cell carcinoma (OSCC) (19).

Likewise, the HCMV play important oncomodulatory role in the pathogenesis of (OSCC) in Iran as reported by Saravani et al., (2015) (20).

In regards to oncogenesis, viral agents could have a chance to play a role in tissue carcinogenesis/oncogenesis at either an early or late events or continuously from early to late in such process. Herein, in this study, it was found that the rank of HCMV-related OCC consistently are elevated in later stages of disease comprising of (85.71%).

These results showed that the virus have played a role in late events of the multistep of oral cavity carcinogenesis. Keeping in mind the small sample size enrolled and to individual variability factors related to the individual differences in the visual judgment of the pathologists which could preclude obvious conclusions regarding the exact role of CMV in oral cavity carcinogenesis.

Furthermore, the percentage of viruses in OCC group was found to increase with the proceeding of grading when reaching to undifferentiated grade.

In addition, HCMV contagions may have been resulted to the development of OC carcinomas and possibly has exerted its oncogenic effects in compact with co-factors in implication with this viral infection.

The high rates can be concluded of HCMV in their evident correlation with the OCC grading might point to HCMV's molecular role in the etiology of these cancers that might appear late, along with other important oncogenic viruses; more extensive studies along with inclusion of high cumulative numbers to unravel their exact role in end OCC oncogenesis.

A small size sample enrolled in the studies analyzed has undermined this study's statistical power to identify the impact of these variables under consideration. Furthermore, the imperfection of clinical detailed information of the carcinoma patients (where this analysis included only tissue samples) the current study was also robbed to a good understanding of the true role in OC carcinogenesis of these mixed viral infections and in turn a suggestion raised to obligate an integrated group-work study, at both molecular and virological levels to clarify the role of these factors and many other agents in OC carcinogenesis in this country. In addition, the positive-EBV results recorded in OCC gave a clue that the virus has a role in the late events of OC carcinogenesis process acting in an interplay manner with many other molecular factors because the highest percentage (76.92%) of viruses was found in the advanced grade of OCC.

In this study it is obvious that a tendency to associate the EBV infection raised which pursued the breaking down in the histopathological features of the test OCC tissues, which are an improved level of identification of EBV by the advance of cancer tissue scoring in this research.

In addition this could also indicate that there are more possible effects of EBV contagion, beside the other factors, in the breaking down of the histopathology of OC cancerous tissues which are taken from the studied patients.

The high rate of EBV infections as well as their evident correlation with the differentiation of OCC could point to the role of EBV in these cancers as a molecular attack which probably occurred at a late event, along with other important oncogenic viruses.

However, the authors of this study believe that further studies are required to reveal the EBV role as well as pathogenesis in OCC, that is in link of importance of EBV vaccine which is presently under clinical trial research for a better understanding of the association between EBV infection and OCC initiation and progression.

In regards to EBV and CMV with grading in research, the grade of OCC co-infected with CMV and EBV, the viruses constituted (80.85%) in the advanced stages of differentiation, that means these viruses collaborated with each other to play a vital role in late events of OCC carcinogenesis in association with other genetic or environmental factors assisted in this process.

Table 1: Co-association of CMV and EBV infections with the differentiation of Oral Cavity Carcinoma (OCC)

Studied groups	Diagnosis		Viral infections				Pearson Chi-Square (P-value)
			Negative	HCMV	EBV	HCMV & EBV	
Oral Cavity	Well	N	0	0	3	7	P=0.253

	differentiated	%	0%	0%	23.08%	14.89%	Non Sign. (P>0.05)
	Moderately differentiated	N	2	1	0	2	
		%	33.33%	14.29%	0%	4.26 %	
	Poorly differentiated	N	0	0	1	3	
		%	0%	0%	7.69%	6.38%	
	Undifferentiated	N	4	6	9	35	
		%	66.67%	85.71%	69.23 %	74.47%	
	Total	N	6	7	13	47	
		%	100%	100%	100%	100%	

Coexistence of p27 and p73 Gene Products in Malignant Tumors

Table (2) shows the presence of one or both of p27 and p73 and their co-presence or co-absence in OCC tissues. The co-existence of p27 and p73 were highest in undifferentiated tissues (70.27%) followed by well differentiated group (21.62%) then poor differentiated (5.41%). While the expressions of p73 alone were (6.67%, 13.33 % and 80%, for well differentiated, poor differentiated and undifferentiated grades, respectively). In regards to p27 expression were (80% and 20%) in undifferentiated and poorly differentiated tissues but its expression, it was not revealed in all other grades.Whereas, p73 expressions alone in moderate and poor groups were (0% and 13.33 %), respectively. There are no significant differences according to the results of OCC grading. In regards to co-existence of p73 and p27 it plays a late role in OCC tumorigenesis since their percentages were (75.68%) in later stages of this cancer.

Table 2: Co-presence of p27 and p73 markers in Oral Cavity Carcinoma (OCC).

Studied groups	Diagnosis		P27 & P73				Pearson Chi-Square (P-value)
			Negative	P27	P73	P27 & P73	
Oral Cavity carcinoma (OCC)	Well differentiated	N	2	0	1	8	P=0.081 Non Sign. (P>0.05)
		%	12.5 %	0%	6.67%	21.62%	
	Moderately differentiated	N	3	0	0	1	
		%	18.75%	0%	0%	2.7 %	
	Poorly differentiated	N	0	1	2	2	
		%	0%	20%	13.33 %	5.41%	
	Undifferentiated	N	11	4	12	26	
		%	68.75%	80%	80%	70.27%	
	Total	N	16	5	15	37	
		%	100%	100%	100%	100%	

Correlation between markers in patients with Oral Cavity Carcinoma

There are strong positive relationships between P73 and p27 scores in OCC tissues. As well as, there are strong positive relationships between EBV and HCMV scores. While this relationship was weak negative non-significant between EBV and P27 as noticed in (Table 3).

Table 3: Spearman's rho Statistical Testing to evaluate p27 and p73 scoring & intensities in Relation with HCMV and EBV Infections in Oral Cavity Carcinoma (OCC).

Spearman's rho Correlation		scoring			Intensities		
		P27	P73	HCMV	P27	P73	HCMV
P73	r.	.321**			.576*		
	P-value	.000			.000		
HCMV	r.	.027	.053		-.122	.066	
	P-value	.131	.417		.153	.297	
EBV	r.	-.022	-.014	.395**	.144	-.113	.321
	P-value	.410	.623	.000	.396	.444	.079

*. Correlation is significant at the P < 0.05 level (Significant).

**. Correlation is significant at the P < 0.01 level (Highly Significant).

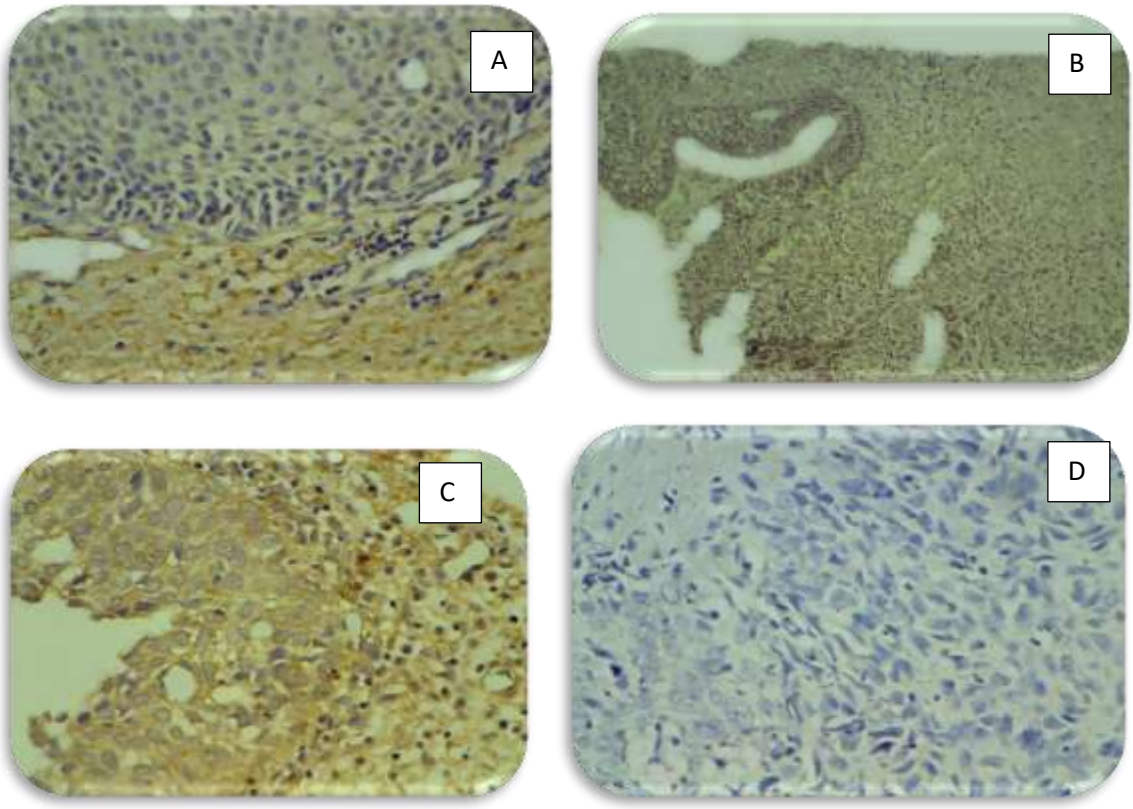

Figure 1: Microscopic image of IHC-p27 positive signals appeared as a brown discoloration. A: OSCC tissue infected with HCMV. B: OSCC tissue infected with EBV. C: OSCC tissue co-infected with HCMV& EBV. D: Negative staining of OSCC tissue.

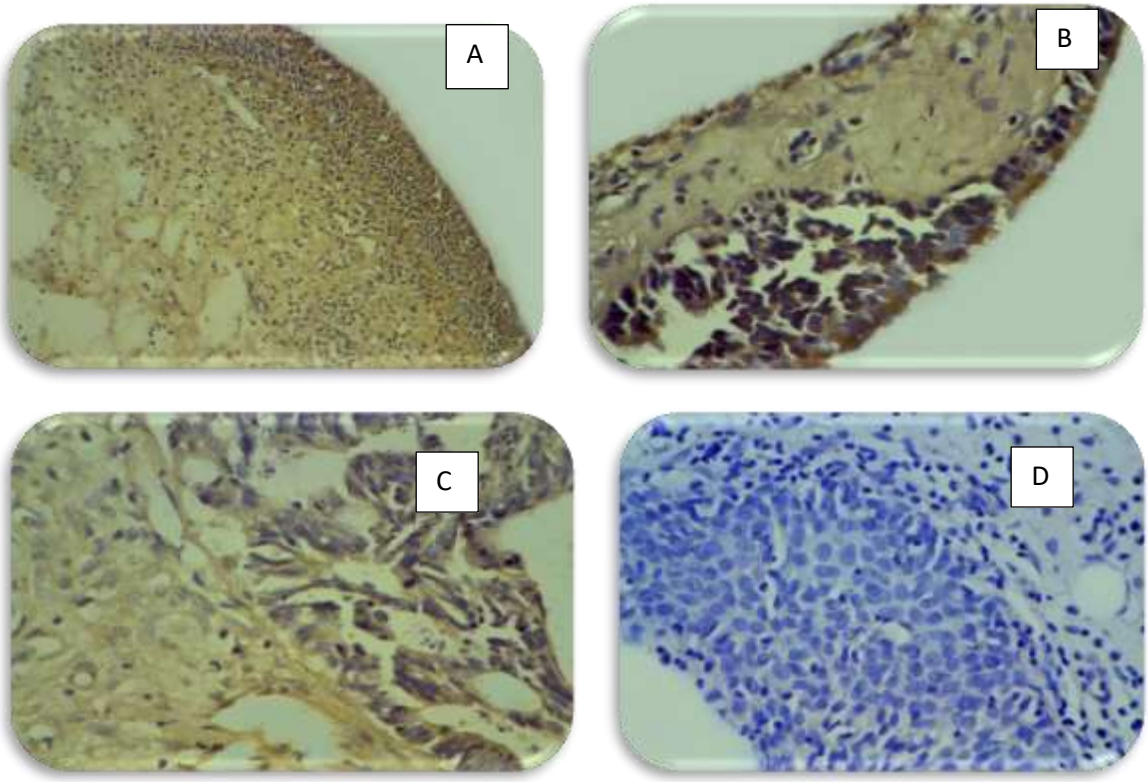

Figure 2: Microscopic appearance of IHC-p73 positive signals appeared as a brown discoloration. A: OSCC tissue infected with HCMV. B: OSCC tissue infected with EBV. C: OSCC tissue co-infected with HCMV&EBV. D: Negative staining of OSCC tissue.

CONCLUSION

Even though there are many different etiological factors of oral squamous cell carcinoma, the viruses are more important. In this study we can conclude that the co-infections of EBV and HCMV may participate together in OCC carcinogenesis as well as, elevation of the expression rates of p27 and p73 gene products in OC malignancies with limited or even no influential regulatory role which was increasing when a malignant tissues infected with two virus together, may indicate mutational events in the genes expressing such proteins. The co-expression of cell cycle proteins and viral genes considerably in OSCC tissues could point for their probable role in either oral pathogenesis or carcinogenesis.

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