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Expression of a cell cycle regulatory protein (P73) correlated with IL-10 levels in HPV-infected gastric cancer patients

Mustafa Kadhem Mallakh[1], Majid Mohammed Mahmood[2], Saad Hasan Mohammed Ali[3]

Department of Medical Laboratory Techniques, Ashur University College, Baghdad, Iraq[1]
Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq[2]
Clinical Communicable Diseases Research Unit, College of Medicine, University of Baghdad, Iraq[3]

ABSTRACT— Gastric cancer is a multifactorial disease in which numerous factors, microbial infections have been shown to contribute to gastric tumorigenesis. HPV is among the well-known causes of cancer infectious agents. HPV is divided into two categories: low-risk HPVs that cause anogenital and cutaneous warts, and high-risk HPVs that cause oropharyngeal cancers as well as anogenital cancers. P73 is a transcription factor that belongs to the p53 gene family, p73 gene produces two types of proteins: full-length isoforms (TAp73), which act as transcription factors, and N-terminal truncated variants (ΔNp73), which lack the TAD, resulting in transcriptionally inactive isoforms that behave as oncogenes in a dominant-negative way. IL-10 is an important regulatory cytokine with anti-inflammatory properties. IL-10 is one of the several important cytokines involved in cancer development and sustenance. The study aims to explore a possible etiological association between high oncogenic-risk HPV genotype (16, 18, 31, 33) DNA and GC, also to evaluate the immunohistochemical expression of p73 and IL-10 proteins in GC tissues. This study involved 84 selected formalin-fixed, paraffin-embedded tissue blocks samples, the collected samples were divided into the following study groups: 54 blocks of GC mass tissues and 30 non-malignant gastric tissues used as a control group for this study. CISH was used to detect HPV DNA16/18 and DNA31/33 in gastric tissues. Regarding the mass GC group, the total percentage of positive HPV16/18 and 31/33 was 44.4%, whereas in the non-malignant group HPV16/18 and 31/33 DNA constituted 80.0%. Also, the total percentage of positive p73-IHC detection was 63%, whereas in the non-malignant group p73 constituted 60%. IL-10 was 74.1% in malignant gastric tissues and 40.0% in non-malignant gastric tissues. These data support previous studies suggesting a role for HPV infection in GC and also provide evidence for an association between HPV infection and p73 with IL-10 proteins expression in GC.

KEYWORDS: Gastric cancer, HPV, Chromogenic in situ hybridization, P73

1. INTRODUCTION

Gastric cancer (GC) was the third most cause of mortality worldwide [1]. Incidence rates vary significantly by geography, with differences of up to 20-fold [2]. In Iraq, GC has a low rate and it is the ninth most common cancer [3].

GC is a multifactorial disease in which numerous factors, both environmental and genetic, impact its development [4]. Microbial infections have also been shown to contribute to gastric tumorigenesis [5], H. pylori and Epstein-Barr virus (EBV) have been recognized as infectious agents, and the potential mechanisms inducing gastric carcinogenesis are well established [6]. Other viruses might play potential roles in gastric carcinogenesis. HPV is among the well-known oncogenic infectious agents [7]. HPV is a

small, double-stranded DNA virus that belongs to the family of Papillomaviridae. It is divided into two categories: low-risk HPVs (LR-HPVs) that cause anogenital and cutaneous warts, and high-risk HPVs (HR-HPVs) that cause oropharyngeal cancers as well as anogenital cancers [8].

P73 is a transcription factor that belongs to the p53 gene family, which also includes p53 and p63. McKeon's group cloned the human Trp73 gene more than two decades ago, and identified its chromosomal position at 1p36, a region frequently deleted in malignancies such as neuroblastoma and other late-stage human cancers [9], thus directly tying p73 to cancer. IL-10 is a key regulatory cytokine with anti-inflammatory activity. It is encoded by the IL10 gene, which is located on chromosome 1 at q31-32. IL-10 was once assumed to be generated only by Th2 cells, but it is now recognized to be produced by a number of cell types [10]. In GC cells, IL-10 regulates a global altered gene expression profile, including metabolic signaling pathways, cell proliferation, and migration. These findings revealed that IL-10 generated by cancer-associated macrophages increased GC proliferation and invasion [11]. In this present study we explore a possible etiological association between high oncogenic-risk HPV genotype (16, 18, 31, 33) DNA and GC, also to evaluate the immunohistochemical expression of P73 and IL-10 in GC tissues.

2. Materials and Methods

2.1 Tissue samples

This study was designed as a retrospective one. It has involved 84 selected formalin-fixed, paraffin-embedded tissue blocks samples which were collected from (Al-Kindy Teaching Hospital, Teaching Laboratories and Gastroenterology and hepatology teaching hospital). The age range of the patients was 25–78 years. These tissue blocks were collected during the period extended from January to November 2020 where these blocks were belonging to the last six years (2014, 2015, 2016, 2017, 2018, 2019) and 2020. The collected samples were divided into the following study groups: fifty-four (54) blocks of gastric adenocarcinoma mass tissues and thirty (30) non-malignant gastric tissues used as a control group for this study.

2.2 Methods

Paraffin-Embedded blocks of tissue for this research and control groups have been collected. New sections were made 4µm thick sections were stuck on positively charges slides of the Paraffin-Embedded blocks for Chromogenic in situ hybridization (CISH) test to detect HPV DNA (genotype16/18 and 31/33) and Section for Immunohistochemistry (IHC) to detect P73 and IL-10 proteins.

2.3 Chromogenic in situ hybridization analysis

CISH analysis was based on the ZytoFast kit (HPV 16/18 and 31/33 probes Digoxigenin-labeled/ ZytoVision, GmbH, Br, Ge). The slides were placed in a 70°C hot-air oven overnight for CISH, after which the tissue sections were deparaffinized and treated with graded alcohols according to standard methods, and the details of the processes for performing a CISH reaction with this probe were applied according to the manufacturer's instructions. After that Pipette 10 µl of the probe was put onto each pretreated specimen. Cover specimens with coverslip and then place slides on a hot plate and denature specimens for 5 min at 75°C, transfer slides to a humidity chamber and hybridize for 1 h at 37°C. A positive DNA probe was included in each run of CIHS. This probe contains a complementary sequence that hybridized with a sequence in the tested tissue but not with the viral genome (e.g., human genomic DNA probe). Positive control tissue was prepared from tissues that were previously known to contain the target marker. A negative DNA probe was included in each run of CISH. It contains, all reagents except the DNA probe. Proper use of this hybridization/detection system gave a strong blue signal that will be sent at the specific

site for the hybridization probe in the positive test tissue. Quantification of different molecular markers CISH signal was evaluated under a light microscope at (100X), (400X), and the Oil Immersion (1000X) respectively. The positive cell count was performed at (1000X). CISH was given Scoring percentage and intensity, based on the number of signals and the intensity of positive signals, respectively. The scale of (0-3) was used for relative intensity with 0 corresponding to no detectable CISH reactions and (1, 2, and 3) equivalent to the low, moderate, and high intensity of reaction respectively. The positive cells were counted in the (10) diverse fields of (100) cells to every sample and the rate of the positive cells of the (10) fields were determined to assign cases to (1 of the 3) following score categories Score (I) = 1-25%, Score (II) = 26-50% and Score (III) > 50%.

2.4 Immunohistochemistry analysis

Exposed rabbit-specific antibodies included in the HRP/DAB detection IHC kit had been used for the detection of p73 protein (Cat. Number: ab64261) (Abcam /UK) and IL-10 protein (Cat. Number: ab34843) (Abcam / UK). Slides were deparaffinized and rehydrated. After that, the slides were blocked with protein block and incubated for 1 hour, application of 30-50µ1 of diluted primary antibody done to each slide, and the slides were incubated according to manufacturer' protocol, apply Biotinylated Goat Anti-Polyvalent to cover tissue sections and incubate for 1 hour, then 30µl of chromogenic DAB has been added to (1.5) ml of the DAB- substrate and incubate for (1-10) min. Counterstained was added to the slides for 1 to 2 min and slides were fitted with a mounting medium, covered with coverslips, and exam under a light microscope. Positive control was prepared from the tissues formerly known to that contain the targeted marker in this study gastric carcinoma included as positive control tissues P73 and IL-10 proteins. Negatively control was used for every IHC run by applying an antibody diluent (PBS) instead of the diluted antibody.

3. Statistical Analysis

SPSS statistics program Version 20.0 for windows was used to do the statistical analysis of the current study & Microsoft Excel 2019 for graphics presentation. The usual statistical methods were used to assess and analyze the results. Chi-square test was used to detect the significance between variables of our study. P-value was considered significant when < 0.05.

4. Result

The archival specimens collected in this study were related to gastric patients whom ages were ranged from 25-78 years. The mean age of the patients with GC (54.85 years) whereas non-malignant gastric tissues was (44.06 years), there was a significant relationship between different groups according to age (Table 1). It was found that 51.9% of GC was male, while the rest 48.1% were females, while in non-malignant gastric tissues there were 53.3% males and 46.7% were females, there was no significant relationship between different groups according to gender (Table 2). This study revealed that stage I occurs in 11.1%, stage II occurs in 59.3%, stage III occurs in 18.5% and stage IIII occurs in 11.1% of GC, there was a significant relationship between different groups according to stages (Table 3). The grading of the GC group in the present study revealed that the well-differentiated group constituting 37.0%, while 51.9% have a moderately differentiated grade, and the poorly differentiated grade was observed in 11.1% of GC group, there was no significant relationship between different groups according to their grade (Table 4).

Regarding the mass GC group, the total percentage of positive HPV16/18–CISH detection was 44.4%, whereas in the control group HPV16/18 DNA constituted 80.0%. Also, the total percentage of positive HPV31/33–CISH detection was 44.4%, and in the control group, HPV31/33 DNA constituted 80.0%. Statistically, there was a significant relationship between different groups according to HPV prevalence (Table 5). Histopathological features were studied between positive and negative HPV with GC. It was

found the positive results of CISH reactions of HPV16/18 and 31/33 according to tumor stage and gender of GC tissues, where HPV16/18 was found 33.3% in stage I, 43.8% in stage II, 40.0% in stage III, and 66.7 % in stage IIII of HPV16/18 and found a positive result in male 42.9% and female 46.2%. While HPV31/33 was found 33.3% in stage I, 43.8% in stage II, 40.0% in stage III, and 66.7 % in stage IIII and found a positive result in male 50.0% and female 38.5%. There was no significant relationship between different groups according to tumor stages and gender (Table 6) (Table 7).

In the GC group, the total percentage of positive p73-IHC detection was 63.0%, whereas in the control group p73 constituted 60.0%. We observed slightly predominantly nuclear staining (51.2) than cytoplasmic staining (48.7) for p73 in malignant GC. Statistically, there was no significant relationship between different groups according to p73 prevalence (Table 8). Also, there was a relationship between positive and negative p73 with GC. It was found the positive results of IHC reactions of p73 according to tumor stage of GC tissues were 66.7% in stage I, 62.5% in stage II, 80.0% in stage III, and 33.3 % in stage IIII. There was no significant relationship between different groups according to stages (Table 9).

IL-10 was identified by Immunohistochemistry technique in 74.1% of malignant gastric tissues and in 40.0% of non-malignant gastric control tissues. We observed predominantly cytoplasmic staining (65.0) than nuclear staining (35.0) for IL-10 in malignant GC. Statistically, IL-10 was significantly different in relation to the gastric groups ($P<0.05$) (Table 10). It was found the positive results of IHC reactions of IL-10 according to tumor stage of GC tissues were higher in stage IV (100.0%) followed by stage III (80.0%), then stage II (68.8%), and finally in stage I (66.7%) (Table 11).

Finally, there was a relationship between positive and negative p73 with HPV16/18 and 31/33 DNA. It was found the positive results of CISH reactions of p73 58.3% in positive HPV16/18, 31/33 cases, and 66.7% in negative HPV16/18, 31/33 cases. Also, there was a relationship between IL-10 with HPV16/18 and 31/33 DNA. It was found the positive results of CISH reactions of IL-10 were 66.7% in positive HPV16/18 and 31/33 cases, and 80% in negative HPV16/18 and 31/33 cases. There was no significant relationship between different groups (Table 12) (Table 13).

Table 1: Distribution of study groups according to their age

	Malignant tissues			Non-malignant tissues			P-value
	Mean	N	%	Mean	N	%	
<=50 year	54.85	20	37%	44.06	22	73.3%	0.024
51+ year		34	63%		8	26.7%	
Total	54			30			84

Table 2: Gender distribution of the total patients according to their sites of malignancy

Site of tumors		Malignant tissues		Non-malignant tissues		P-value
		N	%	N	%	
Gastric	Male	28	51.9%	16	53.3%	0.417
	Female	26	48.1%	14	46.7%	

Table 3: Stages distribution of the patients with GC

Stage	Gastric cancer		P-value
	N	%	

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I	6	11.1%	0.014
II	32	59.3%	
III	10	18.5%	
IIII	6	11.1%	

Table 4: Distribution of GC group according to their grading.

Grade		**Gastric**		**P-value**
		N	**%**	
	Well	20	37.0%	0.460
	Moderate	28	51.9%	
	Poor	6	11.1%	

Table 5: Expression of HPV16/18 and 31/33 DNA in GC group

	Malignant tissues		**Non-malignant tissues**		**P-value**
	N	**%**	**N**	**%**	
HPV 16/18 +ve	24	44.4%	24	80.0%	0.018
HPV 16/18 –ve	30	55.6%	6	20.0%	
HPV 31/33 +ve	24	44.4%	24	80.0%	0.049
HPV 31/ 33 –ve	30	55.6%	6	20.0%	

Table 6: HPV16/18 and 31/33 distribution according to stage distribution

HPV 16/18 and HPV 31/33	**Stage**								**P-value**
	I		**II**		**III**		**IIII**		
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	
Positive	2	33.3%	14	43.8%	4	40.0%	4	66.7%	0.851
Negative	4	66.7%	18	56.2%	6	60.0%	2	33.3%	

Table 7: HPV 16/18 and 31/33 distribution according to gender distribution

		Gender				**P-value**
		Male		**Female**		
		N	**%**	**N**	**%**	
HPV 16/18	**Positive**	12	42.9%	12	46.2%	0.863
	Negative	16	57.1%	14	53.8%	
HPV 31, 33	**Positive**	14	50.0%	10	38.5%	0.704
	Negative	14	50.0%	16	61.5%	

Table 8: p73 distribution according to group

	Group				**P-value**
	Malignant tissues		**Non-malignant tissues**		
	N	**%**	**N**	**%**	
P 73 +ve	34	63.0%	18	60.0%	0.850
P 73 –ve	20	37.0%	12	40.0%	

	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**P-value**
P 73 nuclear	51.24	32.41	5.00	98.00	37.22	26.47	5.00	80.00	0.277
P 73 cytoplasm	48.76	32.41	2.00	95.00	62.78	26.47	20.00	95.00	0.277

Table 9: p73 distribution according to stage distribution

P 73	**Stage**								**P-value**
	I		**II**		**III**		**IIII**		
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	
Positive	4	66.7%	20	62.5%	8	80.0%	2	33.3%	0.621
Negative	2	33.3%	12	37.5%	2	20.0%	4	66.7%	

Table 10: IL-10 distribution according to group

	Groups				**P-value**
	Malignant gastric tissues		**Non-malignant control tissues**		
	N	%	N	%	
IL 10 +ve	40	74.1%	12	40.0%	0.047
IL 10 –ve	14	25.9%	18	60.0%	

	Mean	SD	Min	Max	Mean	SD	Min	Max	**P-value**
IL-10 nuclear	35.0	7.07	64.45	42.21	47.5	52.0	41.67	46.46	0.723
IL-10 cytoplasm	65.0	7.07	35.55	42.21	52.5	52.0	58.33	46.46	0.723

Table 11: IL-10 distribution according to stage distribution

IL-10	**Stage**								**P-value**
	I		II		III		IV		
	N	%	N	%	N	%	N	%	
Positive	4	66.7%	22	68.8%	8	80.0%	6	100.0%	0.691
Negative	2	33.3%	10	31.2%	2	20.0%	0	0.0%	

Table 12: Relationship between positive and negative P73 with HPV16/18 and 31/33 DNA

Gastric			**HPV 16/18**		**Total**	**P-value**
			Positive	**Negative**		
p73	**Positive**	**N**	14	20	34	0.706
		%	58.3%	66.7%	63.0%	
	Negative	**N**	10	10	20	
		%	41.7%	33.3%	37.0%	
HPV 31/33						
p73	**Positive**	**N**	14	20	34	0.656
		%	58.3%	66.7%	63.0%	
	Negative	**N**	10	10	20	
		%	41.7%	33.3%	37.0%	

Table 13: Relationship between positive and negative IL-10 with HPV16/18 AND 31/33 DNA

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Gastric			HPV 16/18		Total	P-value
			Positive	Negative		
IL-10	Positive	N	16	24	40	0.662
		%	66.7%	80.0%	74.1%	
	Negative	N	8	6	14	
		%	33.3%	20.0%	25.9%	
HPV 31/33						
IL-10	Positive	N	16	24	40	0.662
		%	66.7%	80.0%	74.1%	
	Negative	N	8	6	14	
		%	33.3%	20.0%	25.9%	

5. Discussion

Some authors believe that there may be a correlation between HPV infection and the development of GC similar to that found for EBV. However, the role of HPV in GC has not been yet extensively studied. Therefore, some recent papers have aimed at demonstrating a correlation between HPV and GC. Unfortunately, those papers provided contradictory data [12].

Demographic data including age, sex, stages, and grade in patients with GC in the present study are comparable to those reported by [13], which indicate that most ages of the patients with GC were higher than 50 years, the cancer incidence rate among men is slightly higher than women, more common GC patients were moderately differentiated (52.3%).

In the present study, HPV16/18 DNA and 31/33 were detected in (44.4%) in GC by using the CISH technique in the Iraqi population that is equivalent to previously reported in studies from other countries using various experimental techniques but in different percentages. One study in China conducted by demonstrated a rate of (52%) overexpression of each of HPV16 and HPV18 in GC patients [14]. Another study conducted in Turkey by [15] found that among gastric adenocarcinoma samples, 38% were HPV DNA positive by using real time PCR. In our GC patients, we have not observed a correlation of HPV infection with gender and tumor stages these results were in agreement with [16]. Also, we found HPV16/18 DNA and 31/33 detected in (80.0%) in non-malignant gastric tissues, these result agreement study conducted by [17] that found 27 out of 35 laryngeal benign lesion patients (77%) had hrHPV types, higher than the percentage of prevalence of hrHPV in laryngeal squamous cell carcinoma patients (75%).

The present study revealed overexpression of p73 in GC tissues and non-malignant gastric tissues (63% and 60% respectively), but there is no significant correlation between them, these results occur in the previous study but in different percentages, where one study conducted by [18] found that the positive rate of p73 protein expression in the gastric carcinoma group was 62.5% [19] reported a higher 75.0% frequency of p73 expression in GC. Few studies are available in the English literature that has analyzed the clinicopathological correlation of p73 expression in GC, in the present study we observed, there is no significant correlation between p73 expression and GC stage, these results agree with the study of [20] that did not detect any statistical differences in the positivity of p73 concerning tumor stage. High p73 levels are a prognostic factor correlating with poorer survival compared to undetectable p73 [21]. The functional significance of p73 upregulation remains unclear, but it may, in combination with other aberrantly expressed proteins, stimulate cell growth. It has recently been reported that p73 synergizes with the proto-

oncogene cJun and augments the transcriptional activity of AP1, a transcriptional complex that contributes to transformation and tumor aggressiveness [22].

The present study revealed an IL-10 up-regulation in GC tissues in comparison to non-malignant gastric tissues. Because of the high levels of IL-10 discovered in gastric carcinoma patients in this study, it's possible that it's inhibiting anticancer responses. IL-10 seems to have more pro-tumor properties, where the inhibitory effect of IL-10 on Th1-cytokine production, particularly IL-12p70, through engaging apoptosis and encouragement of cell growth, might explain its pro-tumor features. Furthermore, because certain tumor cells release IL-10, there is evidence that tumor-infiltrating lymphocytes inside the tumor mass are ineffective. IL-10 release is one of the strategies through which tumor cells escape immune surveillance, which might explain the significant increase in IL-10 levels ($p<0.05$) in our study [23]. Our study revealed high expression of IL-10 in the advanced stages of GC, which led us to believe that IL-10 may be good for being used as a marker to distinguish the advanced stages of GC [24].

Up to our best knowledge, no previous studies investigated the association between p73 expression and HPV infection in GC were found. Since this study is the first in this respect. Therefore the results in this study will be discussed and interpreted for an analogy to other cancers. [25] occurred overexpression of p73 proteins have been reported in HPV-positive oropharyngeal cancers as well as [26] found that the levels of p73 are elevated in HPV-positive cells and knockdown impairs HPV genome amplification. In our study, there is a relationship between HPV and p73 expression where p73 is critical for the HPV life cycle, which specific downstream targets of p73 are important for regulating the HPV life cycle are still unclear and subject to future analysis [27]. The activator TAp73 has been reported to increase the expression of p53 responsive genes while ΔNp73 acts as a repressor and both are likely important for HPV pathogenesis [28].

Also, there is an association between IL-10 expression and HPV infection in GC, with thirty percent of HPV-infected GC tissues, having IL-10 expression. Many theories may be used to explain this expression, including the following: The central roles of various cytokines in antimicrobial immunity and inflammation make them candidates for being genetic host markers in evaluating individual susceptibility to GC development, where they may influence the risk of developing GC by altering the quality and vigor of inflammatory responses produced by the host after exposure to various environmental or infectious triggers, as seen in our study in the cases of GC that is infected with HPV [29]. Another hypothesis is that the HPV E2, E6, or E7 proteins will increase IL-10 expression in cancer by identifying the elements and transactivating the IL-10 gene [30].

In conclusion, these data support previous studies suggesting a role for HPV infection in GC and also provide evidence for an association between HPV infection and p73 with IL-10 expression in GC, this could indicate an important role of these molecules in gastric carcinogenesis. However, this observation needs to be substantiated with additional studies analyzing a larger number of individuals.

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