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Original Article

Immunomolecular Investigation of Human Papillomavirus Genotypes (16, 18) and P63 Expression in Patients with Malignant and Non-malignant Colorectal Tumors

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Abstract

Cancer of the colon (colorectal cancer, or CRC) is the third most frequent malignancy in the world and the fourth leading cause of cancer-related death. Recent research has focused on the link between high-risk human papillomavirus (HPV) infections and the onset/development of several different types of cancer in humans. As a result, scientists are now paying more attention to HPV and CRC. In a variety of malignant tumors, P63 is overexpressed. This includes non-Hodgkin lymphoma and breast carcinoma, as well as lung, bladder, and prostate cancers. However, in accordance with the existence of many P63 isoforms in malignant tumors, the actions of P63 in these malignancies remain a source of debate. P63 immunohistochemistry expression in CRC tissues is being investigated as a possible etiological link between high-risk HPV types and CRC. This retrospective study intended to investigate if there was an etiological link between high-risk HPV types and CRC. It has utilized 92 chosen formalin-fixed and paraffin-embedded tissue block samples. The collected samples were divided into 62 blocks of colorectal adenocarcinoma mass tissues and 30 non-malignant colorectal tissues used as a control group. Chromogenic in situ hybridization (CISH) was employed to discover HPV DNA16/18 in colorectal tissues. The overall proportion of positive HPV16/18 DNA- CISH detection in the mass CRC group was 44.4%, whereas HPV16/18 DNA was obtained at 80.0% in the non-malignant control group. The overall proportion of positive P63-ISH detection in the CRC group was also 70.4%, whereas P63 was 73.3% in the non-malignant control group. The link between HPV infection and P63 expression in CRC might point to the importance of these molecules in the progression of CRC.

Keywords: Chromogenic in situ hybridization, Colorectal cancer, HPV, P63

1. Introduction

Colorectal cancer (CRC) is the third most frequent malignancy worldwide and the fourth leading cause of cancer-related mortality (1). The incidence of CRC varies substantially by location. Industrialized nations account for around 55% of CRC cases, whereas Africa and Asia have the lowest prevalence (2). CRC is more likely to develop in people with ulcerative colitis than in the general population. In a small percentage of CRC instances, genetic predisposition plays a role, although the great majority of tumors are sporadic and not hereditary. High-risk human papillomavirus (HPV) infections have been associated with the onset and progression of various human carcinomas (3); therefore, researchers have begun to pay attention to the link between HPV and CRC in recent years.

HPV is a sexually transmitted non-enveloped epitheliotropic double-stranded DNA virus. When

HPVs infect epidermal or mucosal epithelial cells, they can develop into cancer (both benign and malignant) (4). HPV and malignancies of the cervix uteri, penis, vulva, vagina, anus, and oropharynx have been shown to have a strong causative link (5). HPV is a sexually transmitted non-enveloped epitheliotropic double-stranded DNA virus. When HPVs infect epidermal or mucosal epithelial cells, they can develop into cancer (both benign and malignant) (6). According to several studies, HPV16 was discovered in more than 50% of HPV-positive colon cancer tissues, implying that the high rate of HPV infection is linked to the occurrence of local CRC (7). Other studies showed that 14%-84% of colorectal neoplasia is HPV DNA positive (8). Numerous studies have discovered HPV DNA in CRC and adenomatous polyps by the use of polymerase chain reaction (PCR) and in situ hybridization, whereas others have found little or no HPV DNA in these cancers or polyps (9). As a result, the link between CRC and HPV infection is still debatable.

Tumor protein 63 (P63) is a transcription factor of the P53 gene family encoded by the TP63 gene on chromosome 3q28. Genes like basal layer keratins and cell cycle regulatory genes are regulated by P63, which is found in the ectoderm and its related structures and tissues (10). The P63 gene encodes at least six different transcripts (TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ) with transactivation (TAp63) or negative effects (ΔNp63) on the P53 reporter genes (11). P63 is overexpressed in some malignant tumors, such as breast carcinoma (12), lung cancer (13), bladder cancer (14), prostate cancer (15), ovarian cancer (16), and non-Hodgkin lymphoma (17). The actions of P63 in malignant tumors are still debated due to the occurrence of several P63 isoforms. This study investigated a possible etiological link of HPV16/18 DNA with high oncogenic risk and CRC, as well as the immunohistochemistry expression of P63 in CRC tissues.

2. Materials and Methods

2.1. Tissue Samples

This retrospective study included 92 formalin-fixed and paraffin-embedded tissue block samples taken from patients undergoing biopsies and preserved at multiple Baghdad Hospitals' Histopathology Laboratories (Al-Kindy Teaching Hospital, Histopathology Laboratories in the Teaching Laboratories and Gastroenterology and Hepatology Teaching Hospital, Baghdad, Iraq). The patients ranged in age from 20 to 85 years old. The tissue blocks were gathered through the time extended from January to November 2020, and these blocks belonged to the last six years (2014-2019) and 2020. The collected samples were divided into 62 blocks of colorectal adenocarcinoma mass tissues and 30 non-malignant colorectal tissues used as a control group for this study.

2.2. Methods

Paraffin-embedded blocks of tissue for this study and control groups have been gathered. New sections were made, and 4-µm-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 a section for immunohistochemistry (IHC) to detect P63 proteins.

2.3. Chromogenic in Situ Hybridization Analysis

CISH analysis was based on the ZytoFast kit (HPV 16/18 probe Digoxigenin-labeled/ZytoVision, GmbH, Germany). The slides were heated to 70˚C in a hot-air oven overnight so that CISH could be performed on them. Once the tissue slices had been deparaffinized and treated with graded alcohols in line with conventional procedures, the CISH reaction was performed using this probe in accordance with the manufacturer's instructions. After that, a pipette of 10 µl of the probe was put onto each pretreated specimen. The specimens were then covered with a coverslip, and slides were placed on a hot plate and denature specimens for 5 min at 75˚C. Following that, the slides were transferred to a humidity chamber and hybridized 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). It was necessary to create positive control tissue from tissues that had previously been shown to contain the target marker. A negative DNA probe was included in each run of CISH. It contains all reagents, except for the DNA probe. This hybridization/detection device produced a strong blue signal that was transmitted to the specific place for the hybridization probe in the positive test tissue when it was used properly. The CISH signal of several molecular markers was assessed under a light microscope at distinct magnifications of 100× and 400 ×. Moreover, the oil immersion was estimated at 1000×, and the positive cell count was performed at 1000×. CISH was given a scoring percentage and intensity based on the number of signals and the intensity of positive signals. No detectable CISH responses were assigned to a score of 0; however, 1, 2, and 3 were assigned to scores representing the relative strength of the reaction. A total of 10 fields of 100 positive cells were used to count each sample's positive cells, and the positive cell rate in each of those 10 fields was calculated to assign cases to one of the score categories of I (1%-25%), II (26%-50%), and III (greater than 50%).

2.4. Immunohistochemistry Analysis

Exposed rabbit-specific antibodies included in the HRP/DAB detection IHC kit had been used for the detection of the P63 protein (Cat. Number: ab64261) (Abcam/UK). Slides were deparaffinized and rehydrated. After that, the slides were blocked with protein block and incubated for 1 h, and 30-50 µ1 of the diluted primary antibody was applied to each slide, and the slides were incubated according to the manufacturer's protocol. Biotinylated goat anti-polyvalent was also applied to cover tissue sections and incubated for 1 h. Subsequently, 30 µl of chromogenic DAB has been added to 1.5 ml of the DAB-substrate and incubated 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 examined under a light microscope. Positive control was prepared from the tissues formerly known to contain the targeted marker in this study. Colorectal carcinoma included as positive control tissues P63 protein. Negatively control was used for every IHC run by applying an antibody diluent (PBS) instead of the diluted antibody.

2.5. Statistical Analysis

Microsoft Excel 2019 was utilized for the graphical presentation, and SPSS software (version 20.0) was used for statistical analysis in the current study. The usual statistical methods were used to assess and analyze the results. The Chi-square test was employed to determine whether or not there was a statistically significant relationship among the variables in our study. A *P*-value less than 0.05 was considered statistically significant.

3. Results

The patients who participated in this study were within the age ranges of 20-85 years, and the archival specimens gathered in this study were connected to them. Furthermore, the mean age of the patients with CRC was estimated at 52.6 years, whereas that of those with non-malignant colorectal tissues was obtained at 42.7 years. There was a significant relationship between the groups regarding age (Table 1).

Table 1. Distribution of the study groups according to age

	Malignant tissues			**Non-malignant tissues**			***P*-value**
	Mean	**N**	**%**	**Mean**	**N**	**%**	
<=50 year	52.6	26	41.9	42.7	24	80	0.015
51+year		36	58.1		6	20	
Total		62			30		92

It was found that 58.1% (36/62 tissues) of the CRC patients and 66.7% (20/30 tissues) of those with non-malignant colorectal tissues were male, respectively. Accordingly, there was no significant relationship between the groups in terms of gender (Table 2).

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	%	
Colorectal	Male	36	58.1	20	66.7	0.355
	Female	26	41.9	10	33.3	

This study revealed that stages I and II occurred in 16.1% (10/62 tissues) and 22.6% (14/62 tissues) of the CRC patients, respectively. Furthermore, stages III and IV were observed in 54.8% (34/62 tissues) and 6.5% (4/62 tissues) of the patients suffering from CRC. There was a significant relationship between the groups in terms of stages (Table 3). The grading of the CRC group in the current investigation revealed that the well-differentiated group constituted 25.8% (16/62 tissues) of the CRC group, while 67.7% (42/62 tissues) of the CRC had a moderately differentiated grade. However, the poorly differentiated grade was observed in 6.5% (4/62 tissues) of the patients. As a result, there was no significant relationship between the groups regarding their grade (Table 4).

Table 3. Stage distribution of the patients with CRC

Stage	Colorectal cancer		*P*-value
	N	%	
I	10	16.1	0.014
II	14	22.6	
III	34	54.8	
IV	4	6.5	

Table 4. Distribution of the CRC group according to their grading

Grade	Colorectal		*P*-value
	N	%	
Well	16	25.8	0.460
Moderate	42	67.7	
Poor	4	6.5	

Regarding the mass CRC group, the total percentage of positive HPV16/18-CISH detection was 51.6% (32/62 tissues), whereas in the control group, HPV16/18 DNA constituted 73.3% (22/30 tissues). Statistically, there was no significant relationship between the groups in terms of HPV prevalence (Table 5).

Table 5. Expression of the HPV16/18 DNA in the CRC group

	Malignant tissues		Non-malignant tissues		*P*-value
	N	%	N	%	
HPV 16/18 +ve	32	51.6	22	73.3	0.111
HPV 16/18 –ve	30	48.4	8	26.7	

Histopathological features were studied between positive and negative HPV with mass CRC. Positive results were found in terms of the CISH reactions of HPV16/18 according to gender and tumor stage of the CRC tissues. The positive results of HPV16/18 were 50.0% and 53.8% in males and females, respectively. Moreover, HPV16/18 was estimated at 80.0% (8/62 tissues) 71.4% (10/62 tissues), 41.2% (14/62 tissues), and 0.0% (0/62 tissues) in stages I-IV, respectively. There was no significant relationship between the groups regarding gender and stages (Tables 6 and 7).

Table 6. HPV 16/18 distribution according to gender distribution

		Gender				*P*-value
		Male		Female		
		N	%	N	%	
HPV 16/18	Positive	18	50.0	14	53.8	0.833
	Negative	18	50.0	12	46.2	

Table 7. HPV16/18 distribution according to stage distribution

HPV 16/18	Stage								*P*-value
	I		II		III		IV		
	N	%	N	%	N	%	N	%	
Positive	8	80.0	10	71.4	14	41.2	0	0.0	0.133
Negative	2	20.0	4	28.6	20	58.8	4	100.0	

In the CRC group, the total percentage of positive P63-IHC detection was 96.8% (60/62 tissues), whereas in the control group, P63 was estimated at 93.3% (28/30 tissues). Furthermore, slightly predominantly nuclear staining (51.4) than cytoplasmic staining (48.6) was observed for P63 in the malignant CRC. Statistically, there was no significant relationship between the groups regarding the P63 prevalence (Table 8).

Table 8. P63 distribution according to the groups

	Malignant tissues				**Non-malignant tissues**				***P*-value**
	N		**%**		**N**		**%**		
P63 +ve	60		96.8		28		93.3		0.592
P63 –ve	2		3.2		2		6.7		
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	***P*-value**
P63 nuclear	51.4	34.2	0.00	95	39.79	26.0	5.00	80.0	0.226
P63 cytoplasm	48.6	34.2	5.0	100	60.2	26.0	20.00	95.0	0.226

In addition, there was a relationship between positive and negative P63 with mass CRC. The positive results of the IHC reactions of P63 according to tumor stage of CRV tissues were obtained at 100.0% (10/62 tissues), 85.7% (12/62 tissues), 100% (34/62 tissues), and 100.0% (4/62 tissues) in stages I-IV, respectively. There was no significant relationship between the groups according to stages (Table 9).

Table 9. P 63 distribution according to stage distribution

P63	**Stage**								***P*-value**
	I		**II**		**III**		**IV**		
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	
+ve	10	100	12	85.7	34	100	4	100	0.315
-ve	0	0.0	2	14.3	0	0.0	0	0.0	

Finally, there was a relationship between positive and negative P63 with HPV16/18 DNA. The positive results of the CISH reactions of P63 were 93.8% and 100% in the positive and negative HPV16/18 cases, respectively. There was no significant relationship between the groups in this regard (Table 10).

Table 10. Relationship between positive and negative P63 with HPV16/18 DNA

Colorectal			**HPV 16/18**		**Total**	***P*-value**
			Positive	**Negative**		
P63	Positive	N	30	30	60	0.325
		%	93.8	100.0	96.8	
	Negative	N	2	0	2	
		%	6.2	0.0	3.2	
Total		N	32	30	62	
		%	100.0	100.0	100.0	

4. Discussion

CRC is a common tumor for which there is currently no recognized etiology. As a result, all risk factors that contribute to its occurrence must be investigated, including the existence of infections, such as HPV (18). Even though some studies failed to find HPV in CRC patients, others found a wide spectrum of HPV infections in CRC patients. However, how HPV influences carcinogenesis in CRC patients is unknown (19). Patients with colorectal carcinoma in the present study had demographic characteristics that were comparable to those reported by Khalil, Al-Hassawi (20) who found that the mean age of the patients with CRC was 53.65 years, the cancer incidence rate among males was higher, compared to females, the more common CRC patients were moderately differentiated (87.7%), and the majority of the patients (54.3%) had stage III disease.

In the current investigation, HPV16/18DNA was found in 51.6% of the CRC patients and 73.3% of the non-malignant colorectal tissues in the Iraqi population that is equivalent to the results of previously conducted studies from other countries using various experimental techniques but in different percentages. One study in the United States was conducted by McGregor, Byrne (21) and found that HPV prevalence in the non-malignant colorectal tumor was 38% and higher than that in the CRC patients (32%) using the PCR technique. Other investigations conducted by Damin, Caetano (9), Młynarczyk, Malejczyk (22), as well as Mlynarczyk-Bonikowska, Muszyński (23) revealed a

high prevalence of HPV in non-malignant colorectal tissues (50%, 69.56%, and 56%, respectively). There are also other studies revealing the presence of HPV in CRC in similar percentages to those in the current study (51%, 52.9%, and 53.84%, respectively) (24-26). In our CRC patients, no relationship of HPV infection with gender was observed, and the females were slightly more infected with HPV than males. These results were in line with the findings of a study conducted by Sun, Wang (27), as well as Picanço-Junior, Oliveira (28). Furthermore, HPV infection was not correlated with the stage of cancer, and HPV prevalence was more frequent in stages I+II, compared to III+IV. These results are consistent with the findings of a study performed by Bernabe-Dones, Gonzalez-Pons (7).

The present study found a high prevalence of P63 in the CRC tissues and non-malignant colorectal tissues (96.8% and 93.3%, respectively); however, there was no significant correlation between them. These results were found in a previous study but in different percentages, where one study conducted by Carneiro, Ramalho (29) viewed P63 expression in 20% of adenoma and 26% of CRC in their series.

In 2012, Guo, Huang (30) discovered that high levels of P63 expression were found in nearly half of all CRC patients. Additionally, Bahnassy, Zekri (31) discovered that adenoma had a 73.3% frequency of P63 expression, compared to 38.8% in adenocarcinoma; moreover, Albasri, Elkablawy (32) discovered the prevalence of P63 in both malignant and non-malignant CRC. P63 expression was found to be slightly more prevalent in the nucleus of malignant CRC in our study, which was the most expression pattern for a protein that normally exhibits strong nuclear staining. These findings were consistent with the results of previous studies that had discovered a nuclear pattern of P63 expression (30, 31), even though in non-malignant colorectal tissues, primarily nuclear P63 staining was found, which was is in line with the results of a study conducted by Albasri, Elkablawy (32). They showed intense cytoplasmic staining of P63 in the CRC and non-malignant cases. In the English-language literature, only a few researchers have examined the clinicopathological relationships between P63 expression and CRC, which were all insignificant. Guo, Huang (30) performed a study on CRC patients and discovered no statistically significant link between pathological tumor stages and P63 expression in the tumors. To the contrary of the findings of the previous study, which revealed an association between tumor stage and the expression of the protein P63, our findings in the current study revealed no significant association between P63 expression and the stage of cancer.

The results of the current investigation revealed positive relationships between HPV16/18 and P63 in CRC. To our knowledge, no previous studies investigated the association between P63 expression and HPV infection in colorectal tumors (whether benign or malignant), and this is the first study of its kind in this area. As a result, the findings of this study will be examined and analyzed in light of their relevance to other malignancies.

In 2009, Shirendeb, Hishikawa (33) showed that P63 was associated with HPV 16 expression in cervical cancer, indicating that HPV 16 prefers squamous epithelial cells and that P63 may contribute to the viral life cycle by suppressing apoptosis via the ΔNp63 isoforms. Citro, Bellini (34) showed that HPV16 E6/E7 expression may influence the transcription of the P63 gene, boosting both the mRNA and protein levels of the P63 fusion protein. Furthermore, the relationship between HPV oncoproteins and ΔNp63α expression has been shown in HPV-positive head and neck cancer cell lines, where the absence of E6/E7 consistently reduced the expression of Np63 protein levels.

Citro, Bellini (34) confirmed that HPV16 E6/E7 expression can regulate ΔNp63α transcriptionally, increasing both its mRNA and protein levels. Moreover, the link between HPV oncoproteins and ΔNp63α expression was confirmed in head and neck cancer HPV-positive cell lines where the lack of E6/E7

consistently decreased ΔNp63α protein levels.

As a consequence, evidence was revealed for a link between HPV infection and P63 expression in CRC, which might imply that these molecules perform a significant function in the progress of colorectal carcinogenesis in humans. This observation, on the other hand, needs to be supported by subsequent investigations including a greater number of participants.

Authors' Contribution

Study concept and design: M. K. M.
Acquisition of data: M. K. M.
Analysis and interpretation of data: M. M. M.
Drafting of the manuscript: S. H. M. A.
Critical revision of the manuscript for important intellectual content: M. K. M.
Statistical analysis: S. H. M. A.
Administrative, technical, and material support: M. K. M.

Ethics

Approval for the research study was obtained from the Ashur University College, Baghdad, Iraq ethics board (project approval number: 4587914)

Conflict of Interest

The authors declare that they have no conflict of interest.

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