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Immuno histochemical Detection Of The Expressed Protein Of P16 Gene In Colonic Adenocarcinomatous Tissues Infected With Epstein-Barr Virus

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Abstract: Background: Epstein-Barr virus (EBV) has been classified among group 1 listof infectious agentsthat is carcinogenic to humans. Some primary colonic cancers are often overexpressing the p16 protein while in contrast; others showed the high incidence of p16 repression by methylation. Objective: The present study was conducted to explore and compare the demo-pathological relation of EBV infection along with p16 expression in normal colonic mucosa, benign colon tumors, and colonic denocarcinoma. Patients and Methods: Seventy (70) colonic mucosal biopsies were enrolled and examined for EBERs and P16 gene expression which were done by chromogenic in situ hybridization (CISH) and immunohistochemical technique (IHC), respectively. These samples were belonged to (30) patients diagnosed with colorectal cancer mass, (25) from benign colon tumors (15) control tissues, which were proved by colonoscopy and histopathological examinations as apparently normal colorectal tissues. Results: Malignant colonic tumors showed positive results of EBERs -CISH detection in 56.7% (17 out of 30 tissues) while in benign colonic tumors were 24 % (6 out of 25 tissues), followed by the apparently healthy colonic control tissues were 20% (3 out of 15 tissues). The present positive p16-IHC results in malignant colonic tumors were 60% (18 out of 30 tissues) while in the benign colonic tumor and apparently healthy colon control tissues were 68% (17 out of 25 tissues) and 20% (3 out of 15 tissues), respectively. Conclusion: it was concluded from this study that the high rates ofEBV- EBERs expression could point to a their possible roles incolonic carcinogenesis, meanwhile, the p16- proteinexpression in colonic adenocarcinoma as compared to benign colon tumors and normal colonic mucosae could played roles in struggling against both EBV infection as well as colonic carcinogenesis.

Keywords: Colorectal, EBERs, EBV, p16-IHC

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1. INTRODUCTION

Colonic cancer is high prevalence and mortality rates and has ranked as one of the most important problems in cancer all over the world In this respect, colorectal cancers in females as well as males and stand for the most 3rd frequent cancer that leads to deaths all over the world [1, 2].

Accumulating evidences have showed that Epstein-Barr virus (EBV) is tightly correlated to nasopharyngeal carcinoma. Additional scattered reports also showed that this virus is linked to other epithelial carcinomas of other primary sites including breast, lung, gastric and colorectal cancers .In addition, it has been reported that the involvement of EBV with some of these orders has relation with the origin [3]. Epstein-Barr virus (EBV), is belongs a type of family called (herpes), in its formation, it has double-stranded DNA genome, which consists of many viral oncogenes, such as antigens [EBNAs], latent membrane proteins [LMP], and EBV-encoded nuclear. The portal of entry of EBV is considered the oropharyngeal epithelium [4].

The interactions of EBV, usually occurs via CD21 receptor with gp350, the entrance of EBV usually happened via HLA class II on B-lymphocytes, which allow into B-lymphocytes. A part from B-lymphocytes, EBV that may targets other human epithelial as well as other hematopoietic cells (like, natural killer cells , granulocytes, and T cells), that may be occurs in a mechanism diverse from the classic CD21 of B cells [5].

Epstein-Barr virus exists in either latent or lytic replication forms. In the lytic duplication of EBV, the viral genome is dramatically amplified inside the nuclei, a process that arresting cell cycle progression and significantly affecting the processes inside the cells [6, 7].

The development of EBV-associated gastric carcinomas has showed major differences from EBV-associated nasopharyngeal carcinoma in terms of viral gene and cell expression [8]. The early stage (5-year survival), the tumors will get to 90%, and a lot of CRC cases in their difficult stages are now noticed with certain markers and symptoms of the disease [9]. In colorectal cancers, the prognostic biomarker studies have clearly focused on the association clinical tests and the formulation of protein in the primary tumors, and the most beneficial is biomarkers of the tissue, blood, and the stool rather than the detection of CRC [10-12].

The aberrant promoter methylation of *p16* as well as *p14*, *p15* genes have allied with incidence, prognosis, and progressing of a lot of cancers, including CRC [13-15]. The strong p16 term was reported in a lot of neoplasias where the alteration of p16 in issues of cancerous was studied through immunohistochemistry [16, 17].

In view of the aforementioned introduction, this research was performed to explore the impact of EBV infection in association with p16 protein expression with some clinic-pathological characteristics of a collection of patients in Iraq that diagnosed with diverse types of colonic cancer.

2. METHODOLOGY

The retrospective study was designed to recruit 70 selected formalin fixed, paraffin embedded blocks in tissue that diagnosed via accompanied records. Sections of 4 mm-thick tissue were prepared and mounted in paraffin embedded, while the other sections of tissue were fixed on positively-charged slides, the slide was used for p16 antigen using (HRP/DAB immuno enzymatic antigen detection system kit which was purchased from (Abcam, UK) for performing the immunohistochemistry technique. One more slide was used for the detection of EBV-EBERs by CISH kit (Zyto Vision GmbH. Fischkai, Bremerhaven. Germany) was performed using digoxigenin-labeled oligo-nucleotides probes that target the Epstein-Bar Virus- EBER RNA.

The statistical analysis was completed using SPSS/Version–23, while the P-value was counted as significant when it is less than 0.05.

3. RESULTS

3.1 Age of the patients with colonic tumors

No noteworthy difference when P larger than 0.05 involving the average age of patients with colonic carcinomas which was (65.6±6.3 years) and benign colonic tumors patients (61.8±14.6 years) as shown in table 1.

Table 1: Age mean of patients with colonic tumors and their healthy control group

Groups	**N**	**Mean**	**Std. Dev.**	**Std. Error**	**Mini.**	**Maxi.**	**Comparison of significant**	
							P-value	**Sig.**
Colonic cancers	30	65.6	6.3	1.32	57	89	0.187	NS
Benign tumors	25	58.9	11.5	2.63	55	83		
Control	15	72.40	4.9	1.66	62	78	-	-
Total	70							

3.2 Histopathological grade description of colonic cancers:

On distributing patients with colonic cancers group according to their grading (table 2), the present results show that well differentiated colonic cancers (grade I) comprise 30.0 %, while it is reasonable (grade II) and weakly (grade III). The distinguished colonic cancers comprise 43.30 %, and 26.70 % respectively.

The statistical calculation revealed no major differences among the grades of colonic cancers ($P > 0.05$).

Table 2: Colonic cancers according to their grades

Grades	**No.**	**%**	**Comparison of significant**	
			P-value	**Sig.**
Well differentiated	9	30	0.283	NS

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Moderately differentiated	13	43.3		(P > 0.05)
Poorly differentiated	8	26.7		
Total	30	100		

3.3 Epstein Barr Virus (EBERs) - Associated Colonic cancers:

Seventeen out of 30 tissues with colonic cancers showed positive chromogenic in situ hybridization reactions (constituting 56.7%), figure 1 and table 3. The benign colonic tumors that show positive were 24%, while, EBERs- CISH detection in the colonic control tissues group constituted 20% (3 out 15) control tissues group. each of these groups are statistically very highly significant (P<0.0001).

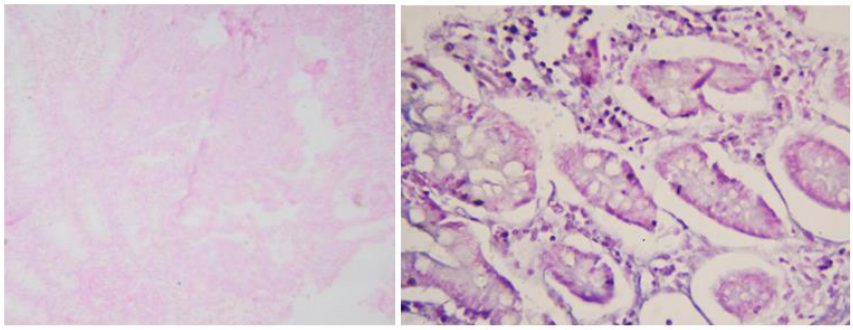

Figure 1: Chromogenic In of EBERS detection: A: Colonic cancer tissue with negative EBERS-CISH reaction (20X). B: Colonic cancer tissue with positive EBERS-CISH reaction (20X).

Table 3: Results of in situ hybridization for detecting EBERS in colonic tumors tissues

Studied groups		EBERS-EBV		Total	Comparison of significant	
		Positive	Negative		P-value	Sig.
Colonic Cancers	N	17	13	30	0.001	Highly Sig. (P<0.01)
	%	56.7	43.3	100		
Benign Colonic Tumors	N	6	19	25		
	%	24	76	100		
Healthy Control Tissues	N	3	12	15		
	%	20	80	100		

3.4 The grading of colonic cancers according to the CISH EBERS- results

61.5% of tissue with colonic cancers was moderate, followed by 55.6% tissues that indicate a differentiated grade, and 50% tissues with poor differentiated grade. The p-value shows important differences, Table 4.

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Table 4: Results of CISH for EBERS detection along with colonic carcinoma grading

Colonic Carcinoma Grades		**EBERS- CISH Reactions**		**Total**	**Comparison of significant**	
		Positive	**Negative**		**P-value**	**Sig.**
Well Differentiated	N	5	4	9	0.02	Sig. (P>0.05)
	%	55.6	44.4	100		
Moderately Differentiated	N	8	5	13		
	%	61.5	38.5	100		
Poorly Differentiated	N	4	4	8		
	%	50	50	100		
Total	N	17	13	30		
	%	56.7	43.3	100		

3.5 Results of P16- IHC Signal Scoring:

Table 5 and figure 2 shows positive signals, of P16-IHC (60 %) from malignant group showed positive signals, included 40% score I (weak), and 13.3 % for score II, while for score III was 6.7 %. 68% for benign group that indicates positive signals including 48% for score I, 12% for score II and 8% for score III. The healthy control group revealed 20% with positive signals including 13.3% score I, 6.7% score II. Statistically, it indicates a high significant difference (p>0.001).

Table 5: Immunohistochemistry of P16 protein in accordance with the scoring of IHC-signal

P16 Protein expression		**Healthy Colonic Tissues (n=15)**		**Benign Colonic Tumor**		**Colonic Cancers (n=30)**		**P**
		N	**%**	**N**	**%**	**N**	**%**	
Negative		12/15	80.0	8/25	32	12/30	40	P<0.004 significant
Positive		3	20	17/25	68	18/30	60	
SCORE	I	2	13.3	12	48	12	40	
	II	1	6.7	8	12	4	13.3	
	III	0	0.0	2	8	2	6.7	
Mean Rank		92.5		89.7		95.3		

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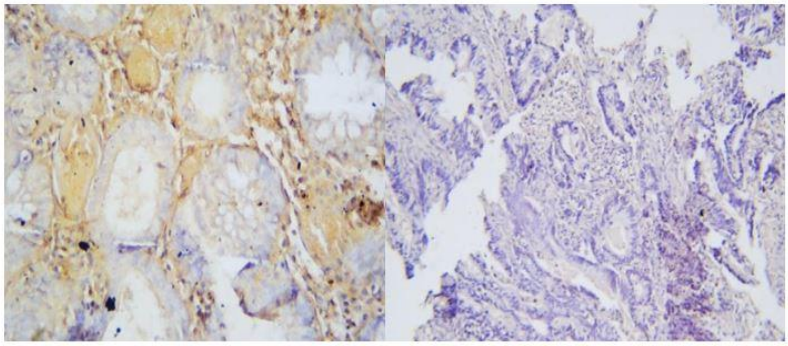

Figure 2: Chromogenic for EBERS, A: Colonic cancer tissue with negative EBERS-CISH reaction (20X), B: Colonic cancer tissue with positive EBERS-CISH reaction (20X).

VI. TheP16-IHC expression along with the histopathological

Table 6 shows the relation of p16-IHC expression to the colonic cancer. The differentiated grade was 66.7% while the moderate was 69.7% and the poor was 37.5%.

Table 6: P16-IHC reactions according to the differentiation of colonic cancers

Colonic Cancer Grade		**IHC Expression of P16 Protein**		**Total**
		Positive	**Negative**	
Well	Count	6	3	9
	% within grade	66.7%	33.3%	100.0%
	% within P16	31.2%	28.6%	29.6%
Moderate	Count	9	4	13
	% within grade	69.2%	30.8%	100.0%
	% within P16	50%	35.7%	44.4%
Poor	Count	3	5	8
	% within Grade	37.5%	62.5%	100.0%
	% within P16	18.8%	35.7%	25.9%
Total	Count	18	12	30
	% within grade	60%	40%	100.0%
	% within P16	100.0%	100.0%	100.0%

3.7 Co-existed expression of EBERS-CISH and P16 –IHC in tissues with colonic tumors

The positive P16-IHC appearance that related with positive EBERS-CISH reaction constituted 64.7%, while the negative reaction of EBERS by CISH technique, the positive P16 expression was 35.3%, and for benign colonic tumors was 56.2%, while it was 43.8% in colonic cancers tissues that showed EBERS-negative reaction, Table 7.

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Table 7: Co-existence of EBERS- and P16 – expressions in tissues with colonic tumors

Studied groups				**EBERS- CISH Reactions**		**Total**
				Positive	**Negative**	
Colonic Cancers	P16 IHC Reactions	Positive	N	11	6	17
			%	64.7	35.3	100
		Negative	N	6	7	13
			%	46.2	53.8	100
		Total	N	17	13	30
			%	56.7	43.3	100
Benign Colonic Tumors	P16 IHC Reactions	Positive	N	9	7	16
			%	56.2	43.8	100
		Negative	N	7	7	14
			%	50	50	100
		Total	N	11	14	25
			%	44	56	100
Healthy Control Tissues	P16 IHC Reactions	Positive	N	0	0	0
			%	0	0	0
		Negative	N	0	15	15
			%	0	100	100
		Total	N	0	15	15
			%	0	100	100

4. DISCUSION

Colorectal cancers are among the most aggressive malignancies that occur at a high incidence in most countries .It was reported that Epstein-Barr virus (EBV) play an oncogenic role in the epithelial cancers, as that happens in colorectal cancers, where several reports concluded a causative link between EBV and colorectal carcinogenesis. EBV cans also being involved in cases such as hyperplasia as well as dysplasia [18]. In addition, previous studies have also explored the contribution of EBV in epigenetic modification in latently infected B lymphocytes, as well as epigenetic regulation of host cell gene expression, where EBV in epithelial cells establishes a typical type II latency program [19].

A Syrian and Iranian studies revealed EBV in 36% and 38% of the cases, respectively [20, 21]. In addition, and by using PCR and IHC assays, global, studies have documented a prevalence of 20%–50% for EBV in colorectal cancers [22-24].

In the current study, seventeen out of thirty (56.7%) colonic cancerous tissues showed positive chromogenic in situ hybridization reactions for EBERs. While benign colonic tumor tissues revealed 24% positive signals for EBERs- CISH detection and 20% in the colonic control tissues group (Table 3 and Figure 1).These findings suggest that EBV may associate

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to the oncogenesis of the examined group of colorectal cancers. Al Moustafa et al., [25] were found in Middle East and North African regionsand by using PCR test that 15% of the examined colorectal adenocarcinoma cases revealed EBV DNA. Liu and co-associates introduced EBV as a carcinogenic factor in the etiology of colorectal cancer where they detected in their study 20% of EBV DNA in 130 colorectal cancers samples that prevailed among men with these cancers [26]. The discrepancies in percentage of EBV detection may partially explained by the small number of the examined cases and by the different sensitivities of techniques used for detection of this virus such as, immunohistochemistry, in situ hybridization, and PCR-based methods, where the later assay is substantially affected by frequent contamination by EBV via the lymphocytes cells that accompanied with colorectal cancer [27]. The $P16^{ink4a}$ protein has negative controlling role on cell proliferation via inhibition of CDK4 kinase to prevent cell proliferation and inhibiting cell cycle at G1 from passing to S phases .The p16 was found to play a crucial role in cell growth *and that p16* -gene genetic variations commonly found in cancer patients [28].

In this study, p16 protein expression was included to unravel its possible role in development of colonic cancers. The positive results of P16-IHC detection was noticed in 60 % of the malignant cases included 40 % with weak score (score I) followed by 13.3 % and 6.7 % with the moderate score (score II) and high score (score III), respectively. According to the result of many previous studies which have demonstrated that development and clinical and pathological characteristics of patients with CRC changed with the expression of p16 protein via homozygous deletion, gene mutation or aberrant methylation of promoter region. Therefore, these could lead to repression of *p16* gene transcription and ultimately inactivation of p16 protein [29]. On the other hand, previous studies have shown that p16- protein overexpression may lead to the loss of CDK4/6-cyclin D via CDK4/6-p16 combination, preventing the cell cycle passage through G1/S restriction point and then repressing cells proliferation [30].

Many studies on $P16^{ink4a}$ protein in CRC significantly showed its higher expression of $P16^{ink4a}$ in normal colorectal tissues than CRCs [31]. On the contrary, Zhao et al. [32] and Lam et al. [33] showed that $P16^{ink4a}$ expression in CRC was higher than both adenoma and normal tissues. Previous reports showed that overexpression of p16 protein played a crucial role in development of CRC and well correlated with their Dukes as well as TNM stages in part of their studied patients and that negative p16 expression have higher chance of lymph node metastasis than those in early stage of CRC [34, 35]. The clinico-pathological roles of p16protein expression in previous studies on colorectal cancers have been studied and showed no agreementregarding these findings. This is likely related to smallnumber of the enrolled patients [35].

The high rates ofEBV- EBERs expression could point to a their possible roles incolonic carcinogenesis, meanwhile, the p16- proteinexpression in colonic adenocarcinoma as compared to benign colon tumors and normal colonic mucosal samples could played roles in struggling against both EBV infection as well as colonic carcinogenesis.

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