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Immunohistochemical Localization of p16 Tumor Suppressor Gene and Bcl-2 Oncogene in Colorectal Tumor Tissues

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Abstract : Background: Colorectal cancers rank fourth in frequency in men and third in women. There is at least a 25-fold variation in occurrence of colorectal cancer worldwide.Bcl-2 is known to inhibit apoptosis and is thought to play a role in colorectal tumor development. In colon cancer, p16 expression is mostly elevated, whereas normal tissues exhibit only little or no protein expression.
Objective: To examine the impact of cellular dysregulation mediated by the concordant protein expressions of P16 & BCL-2 in implicated in colorectal carcinogenesis.
Patients and methods: Seventy-five formalin-fixed, paraffin embedded colorectal tissues were enrolled, among them, 60 biopsies obtained from patients with colorectal carcinomas (30 biopsies from the cancer mass and another 30 biopsies from the marginal tissues of these colorectal cancers) and 15 tissues as control group, which were proved by colonoscopic and histopathological examinations as an apparently normal colorectal tissues. Immunohistochemistry detection system was used to demonstrate the expression of P16 & Bcl-2 genes.
Results: Expression of Bcl_2protein was detected by IHC in 14 cases (46.7%) of the CRC-mass group, 12 cases (40%) of marginal group, and none in control group. A significant differences ($P<0.05$) were found when comparing the mass group with its control group. Expression of P16 protein was detected by IHC in 18 cases (60%) of the CRC- mass group, 10 cases (33.3%) of marginal group, and none in control group. A significant differences ($P<0.05$) were found when comparing the mass group with its control group. **Conclusions:** Our results indicate that the significance prevalence of BCL-2 as well as P16 - expression in colorectal carcinoma could point to an important contributing role of these molecular factors in the development and carcinogenesis of a subset of colorectal cancers.
Key word: CRC, BCL-2, P16,IHC.

Introduction:

Colorectal cancer (CRC) is considered the third main cause of mortality in the world and it is the most common gastrointestinal cancer and the leading of cancer deaths in the United States of America and western countries[1]. Several factors, such as smoking, alcohol use, low rate of fruit and vegetable consumption, obesity, age, family history, red meat consumption, and a lack of physical activity are associated with an increased risk of CRC[2].

The incidence of colorectal cancer varies around the world, where in America, Western Europe, Australia and Japan has the largest rate and in African and Asian countries has the lowest rate[3]. The prognosis of colorectal carcinoma is still being evaluated by histological features[4].

Several studies on molecular biology have been carried out aiming the identification of new prognostic parameters[5]. The factors involved in the cell cycle regulation of growth and cell death mechanisms can affect tumour development[6].

BCL-2 is a human proto-oncoprotein located in the membranes of the nuclear envelope, endoplasmic reticulum, and in the outer membrane of mitochondria[7].

The overexpression of Bcl-2 protein during adenomatous growth suggests that selection of Bcl-2 mediated inhibition of apoptosis is an early event in the development of colorectal tumours[8]. As adenomatous growth and tumour invasion are two different phases of tumour progression, it may be that during the phase of tumor invasion, the apoptotic stresses are different and this necessitates selection of a diVerent means of inhibition of apoptosis (such as p53 mutation). In this case, Bcl-2 function would become redundant and could be lost with no cost to the tumor[9].

Loss of Bcl-2 expression correlates with poor prognosis in both colorectal and non-colorectal tumours. In invasive colorectal tumors,

Bcl-2 expression is an independent prognostic marker. Cancers presenting at Dukes' B stage form a heterogeneous group and it may be possible to separate those cases which have a higher risk of recurrence, and may therefore need adjuvant therapy[10].

The p16 suppressor gene is one of the most commonly studied candidates in the pathogenesis of human neoplasia[11]. P16 gene encodes p16 protein that competes with cyclin D for binding to CDK4[12]. This inhibits the ability of the cyclin D-CDK4 complex to phosphorylate Rb (retinoblastoma) protein, thus causing cell cycle arrest at late G1 phase[13].

In colon cancer, p16 expression is mostly elevated, whereas normal tissues exhibit only little or no protein expression[14]. Protein expression in colorectal cancer seems to resemble p16. Normal tissues showed only little or no cyclin D1 expression[15], whereas the highest levels were found in colorectal carcinomas[16].

The current study is aiming to unravel the P16 & BCL-2 association with colorectal cancer in a group of Iraqi patients.

Materials and Methods:

Study Groups:

This study was designed as a retrospective research; a number of (60) formalin-fixed, paraffin embedded colorectal tissue blocks enrolled in this study which comprised both patients and control samples that their age ranged from 21 to 85 years. These retrospective paraffin-embedded samples were retrieved from the archives of the period from 2011 till 2016 belonging to major hospitals and private histopathological laboratories in Baghdad, Kerbela, Babylon, and Al-Najaf provinces. The diagnoses were based on their accompanied pathological reports of the corresponding patients. These blocks included a group of (60) biopsies from patients who had undergone surgical operation or biopsies for their colorectal cancers (CRC) and (15) colonic tissues (proved by colonoscopy and histopathological examination to be free from any significant pathological changes) were considered as a negative control group for this study. These colorectal tissues were properly subjected to fixation as well as paraffin embedding and used for this research work as an age- and grade- matched groups.

Laboratory methods:

Slide Preparation:

Tissue sectioning was conducted following trimming process of the tissue blocks at the histopathological department of Teaching laboratories / in Al-Yarmooq teaching hospital & in Dr. Israa Mahdi Al-Sudani private histopathology lab. And a second confirmatory histopathological re – evaluation of each obtained tissue blocks was done by a consultant pathologist. One paraffin embedded (4 mm) thick-tissue section was prepared and mounted on ordinary glass slide and stained with hematoxyline and eosin, while other

(4 mm) thick-tissue sections were stuck onto positively charged slide to be used for detection of P16& BCL-2 using Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (Lot. Number: ab64264) that was purchased from (Abcam, UK), an immunoenzymatic antigen detection system for immunohistochemistry techniques, using specific Monoclonal Mouse Anti-BCL2 antibody (BCL2/100) ab117115, was also purchased from (Abcam, UK) and Anti-P16 ARC antibody (EP1551Y) ab51243:, also purchased from (Abcam, UK). The details of methods for performing IHC reaction with these antibodies were conducted according the instructions of that manufacturing company, and were done in the Research Laboratories of the College of Medicine, Al-Mustansiriya University as well as in the private lab.

Histopathological Analysis:

According to the specification of the kit, proper use of this IHC detection system gives an intense brown signal at specific sites of the expression protein in positive test tissues (by using light microscope).

The signal was evaluated under light microscopy using × 100 lens for counting the positive cells. The IHC results were given intensity and percentage scores based on intensity of positive signals and number of cells that gave these signals, respectively.

Positive cells were counted in 10 different fields of 100 cells for each sample and the average percentage of positive cells within the 10 fields was determined. A scale of 0-3 was used for relative intensity with 0 corresponding to no detectable IHC reaction, and 1, 2, 3 equivalents to low, moderate, and high intensity of reaction respectively. Cases were assigned to one of the following percentage score categories: 1%–25% (score 1), 26%–50% (score 2) or > 50% (score 3) [17].

Statistical Analysis:

T test, ANOVA test, and Chi square were applied for statistical examination of results obtained in our research. All these statistical analysis were done by using Pentium-4 computer through the SPSS program (version-19) and Excel application.

Results:

I. Distribution of Patients with Colorectal Cancers and Healthy Control Group According to Their Age .

The archival specimens collected in this study were related to colorectal cancers patients whom ages were ranged from twenty- one years to eighty five years, where their mean age (**53.6**± 15.7 years) was higher than the mean age (45.7 ± 11.2 years) of those enrolled in the apparently healthy control. Statistically, no significant difference ($p < 0.05$) was observed between these groups according to the age (Table 1).

Table (1): Distribution of Colorectal Cancers Patients According to Their Age .

Maximum	Minimum	S.E	S.D	Mean Age	N	Study Groups
85.00	**21.00**	**2.4**	**15.7**	**53.6**	**60**	**Colorectal Cancer**
75.00	**38.00**	**3.2**	**11.2**	45.7	**15**	**Apparently Healthy Control**
(P <0.05)						**Statistical Analysis**

Distribution of colorectal cancer according to site of tumor

The present results revealed a predominance of CRC involvement is the left side of large bowel 46% while the CRC in the right colon forming only 34%. However, The rectum involvement by CRC was 20% (figure 1).

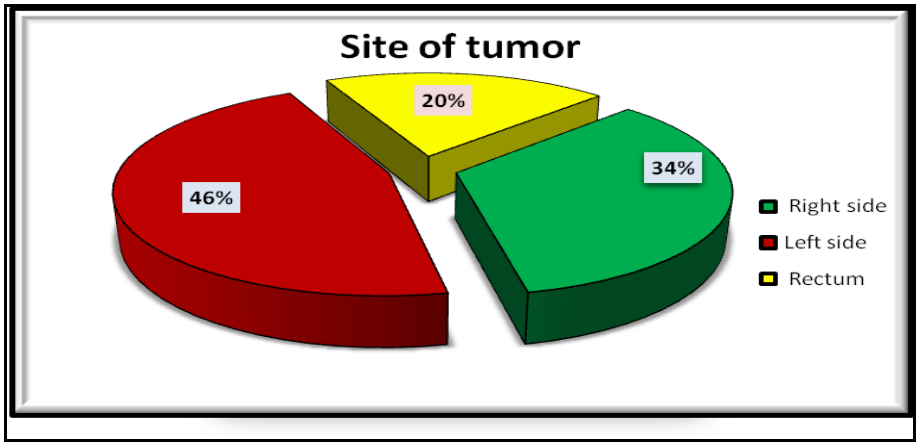

Figure (1): Distribution of colorectal cancer according to site of tumor

Grading of the studied colorectal cancer:

In this study, well differentiated colorectal cancers were seen in 24 cases (40%) including 16 males and 8 females, while 30 cases (50%) (Including 18 males and 12 females) have moderately differentiated grade. Poorly differentiated CRC was seen in only 6 cases which comprising (10%) of total CRC group and among them 4 males and 2 female (Table2). The statistical analysis of grading distribution of colorectal carcinoma shows significant differences ($p<0.001$)among the grades of Colorectal carcinoma.

Table (2): Distribution of colorectal cancers according to their differentiation grades.

Grading of CRC	Gender		Total		P-value
	Male	Female			
	No.	No.	No.	%	
Well differentiated	16	8	24	40	0.03
Moderately differentiated	18	12	30	50	
Poorly differentiated	4	2	6	10	
Total	38	22	60	100	

Results of IHC- Signal Scoring for Bcl_2 protein detection:

Expression of Bcl_2protein was detected by IHC in 14 cases (46.7%) of the CRC- mass group, 12 cases (40%) of marginal group, and none in control group. A significant differences ($P<0.05$) were found when comparing the mass group with its control group. A high percentage of score III (50%; 7 cases) were observed among cases in of the mass group. In the marginal group 7cases (58.3%) revealed score II (Table 3 and Figure 2).

Table (3): Frequency distribution of immunohistochemistry results of Bcl_2 protein according to the signal scoring.

P-value1	Apparently Healthy control tissues (n=15)		Colorectal Marginal Tissues (n=30)		Colorectal Mass Tissues (n=30)		Bcl_2protein signal scoring	
	%	No.	%	No.	%	No.		
0.007	100	15/15	60	18/30	53.3	16/30	Negative	
	0.00	0/15	40	12/30	46.7	14/30	Positive	
	0.00	0.00	16.7	2/12	21.4	3/14	I	Scoring
	0.00	0.00	58.3	7/12	28.6	4/14	II	
	0.00	0.00	25	3/12	50	7/14	III	

Results of IHC reactions for Bcl_2protein according to the Signal intensity:

In all of the studied groups of CRC, the highest percentageof IHC reactions for Bcl_2 protein was found to have strong signal intensity (50%; 7/14) cases of the CRC -mass group,and was found to have moderate signal intensity (58.3% ; 7cases) in the CRC -marginal group. A significant differences ($P<0.05$) were found on comparing the results of IHC reactions according to their intensity among mass & marginal and healthy group (Table 4).

Table (4): Frequency distribution of immunohistochemistry results of Bcl_2 protein according to the signal intensity.

P-value1	Apparently Healthy control tissues (n=15)		Colorectal Marginal Tissues (n=30)		Colorectal Mass Tissues (n=30)		Bcl_2 protein signal intensity	
	%	No.	%	No.	%	No.		
0.04	100	15/15	60	18/30	53.3	16/30	Negative	
	0.00	0/15	40	12/30	46.7	14/30	Positive	
	0.00	0.00	16.7	2/12	28.6	4/14	low	Intensity
	0.00	0.00	58.3	7/12	21.4	3/14	Moderate	
	0.00	0.00	25	3/12	50	7/14	High	

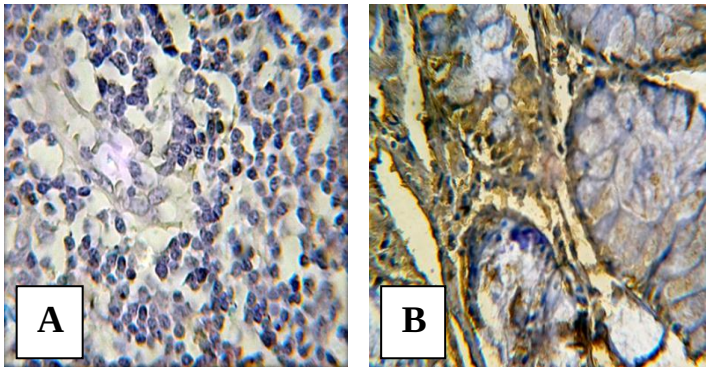

Figure 2: Infiltrative Colorectal Carcinoma Showing the Results of Immunohistochemistry Staining Protein over Expression Using Biotinylated Anti-BCL2 Protein Antibody; Stained By DAB-Chromogen(Brown) and Counter Stained By Mayer's Heamatoxylin (Blue). A- Colorectal Cancer with negative staining for BCL2(40X). B-BCL2-IHC-reaction with high signal score and strong signal intensity (40x).

IV.Results of IHC- Signal Scoring for P16 protein detection:

Expression of P16protein was detected by IHC in 18 cases (60%) of the CRC- mass group, 10 cases (33.3%) of marginal group, and none in control group. A significant differences (P<0.05) were found when comparing the mass group with its control group. A high percentage of score III (44.5%; 8 cases) were observed among cases in of the mass group. In the marginal group 5 cases (50%) revealed score II (Table 5and Figure 3).

Table (5): Frequency distribution of immunohistochemistry results of P16 protein according to the signal scoring.

P-value1	Apparently Healthy control tissues (n=15)		Colorectal Marginal Tissues (n=30)		Colorectal Mass Tissues (n=30)		P16 protein signal scoring	
	%	No.	%	No.	%	No.		
0.009	100	15/15	66.7	20/30	40	12/30	Negative	
	0.00	0/15	33.3	10/30	60	18/30	Positive	
	0.00	0	30	3/10	22.2	4/18	I	Scoring
	0.00	0	50	5/10	33.3	6/18	II	
	0.00	0	20	2/10	44.5	8/18	III	

Results of IHC reactions for P16 protein according to the Signal intensity:

In all of the studied groups of CRC, the highest percentageof IHC reactions for P16 protein was found to have strong signal intensity (61.1%; 11/17) cases of the CRC -mass group, and (60% ; 6/10 cases) in the CRC -marginal group.A significant differences (P<0.05) were found on comparing the resultsof IHC reactions according to their intensity among mass & marginal and healthy group (Table 6).

Table (6): Frequency distribution of immunohistochemistry results of P16 protein according to the signal intensity.

P-value1	Apparently Healthy control tissues (n=15)		Colorectal Marginal Tissues (n=30)		Colorectal Mass Tissues (n=30)		P16 protein signal intensity	
	%	No.	%	No.	%	No.		
0.04	100	15/15	66.7	20/30	40	12/30	Negative	
	0.00	0/15	33.3	10/30	60	18/30	Positive	
	0.0	0/2	10	1/10	16.7	3/18	low	Intensity
	0.00	0/2	30	3/10	22.2	4/18	Moderate	
	0.00	0/2	60	6/10	61.1	11/18	High	

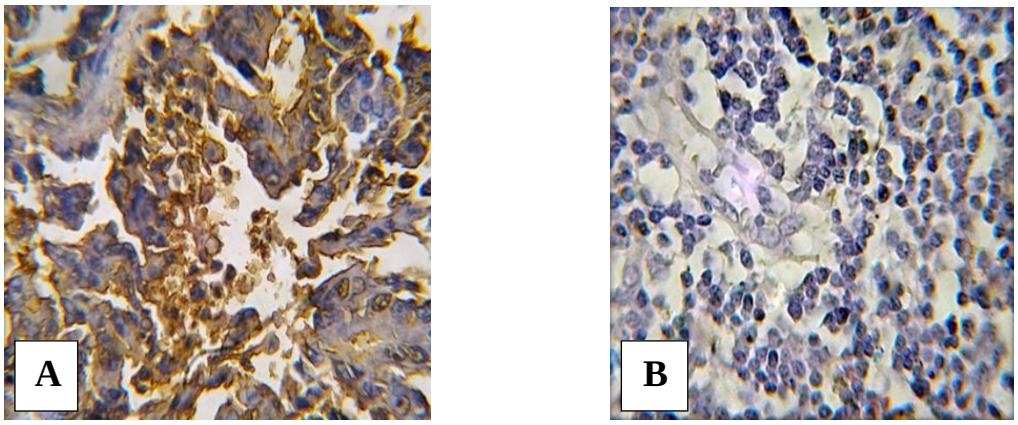

Figure (3): The Results of Immunohistochemical Staining of Total P16 Gene Expression in CRC Using Biotinylated -Labeled Anti- Total P16 Protein Antibody, Stained by DAB-Chromogen (Brown) and Counter Stained By Mayer's Hematoxyline (Blue).A. **colorectal cancer with** Positive**Total P16 –IHC reactions(10X) B. Negative Total P16 –IHC reaction with low score and high signal intensity (10X).**

Discussion:

Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries[18] .Colorectal cancer results from the accumulation of multiple genetic and epigenetic changes leading to the transformation of colon epithelial cells into invasive adenocarcinomas[19].

Only 3-5% of all CRCs are caused by hereditary factors, while the remainder of CRC's being sporadic. The colorectal carcinogenesis is a multi-step/multi-factorial process, where the association between infections by some bacterial and viral agents with CRC was made since several decades ago[20].These results also reflect that age could be an important risk factor affecting colorectal epithelial tissues in favor of tumor changes. In general, the age distribution of the population is considered the most important factor determining the overall incidence of CRC[21].

In the Western world, the incidences of colon and rectum cancers increase at the ages between 50 and 80. In the present study, the mean age of all CRC cases was 56.9years showing that CRCs occur in earlier ages in this population compared to the Western populations. However, the mean survival age of this population is relatively low (70-75 years) and this may explain the decreased incidence of CRC in the elderly[22].

Conventional adenocarcinoma is characterized by glandular formation, which is the basis for histologic tumor grading. In well differentiated adenocarcinoma >95% of the tumor is gland forming. Moderately differentiated adenocarcinoma shows 50-95% gland formation. Poorly differentiated adenocarcinoma is mostly solid with <50% gland formation. In practice, most colorectal adenocarcinomas (~70%) are diagnosed as moderately differentiated. Well and poorly differentiated carcinomas account for 10% and 20%, respectively[23].

The WHO also suggests dividing CRCs into low grade (G1 and G2) and high grade (G3 and G4) categories. The diagnosis of G3 and G4 is relatively consistent, but differentiation between G1 and G2 is associated with a more significant degree of inter observer variability[24].

The well differentiated adenocarcinoma was the most frequent type in this study forming 52.3% of the cases. Likewise, [24] revealed consistent percentages of their studied CRC grading to our results where well differentiated tumors have > 95% glandular structures and are designated grade 1 (G1), moderately differentiated tumors with 50% to 95% gland formation are grade 2 (G2), poorly differentiated tumors with 5% to 50% gland formation are grade 3 (G3) .

There is now solid evidence that a series of genetic alterations in both dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of human colorectal cancer[18].

The overexpression of Bcl-2 protein during adenomatous growth suggests that selection of Bcl-2 mediated inhibition of apoptosis is an early event in the development of colorectal tumors[10]. High levels and aberrant patterns of Bcl-2 expression have been reported in colorectal, lung, gastric, renal and other cancers[25].

In the current study , the positive result of BCL2-IHC detection where 45.2% from malignant group, while, in the benign group revealed 68.75%.The healthy control group revealed 10% positive signals which represented (1 out of 10 cases) in this group (Figure 4-12)**.**However, in colorectal cancer, immunohistochemical positivity is found in up to 42.85% of in situ carcinomas, which suggests ,the bcl-2 protein is known for postponing programmed cell death, by inhibiting apoptosis, propagating cell division and potentially contributing towards tumor growth[26].This results agreement with our study.

Sinicrope *et al*[127] reported the first data concerning the importance of bcl-2 in colorectal tumorigenesis: in 71% of adenomas and in 67% of adenocarcinomas, bcl-2 immunoreactivity was detected.Similar results have been reported in other studies although the reduction of bcl-2 expression in carcinomas compared with adenomas was more apparent[28].

There may also be a loss of expression with loss of tumor differentiation15 and it would appear that the role of Bcl-2 is probably more important in the early development of colorectal tumours than in later tumour progression[10]. This result supports a functional role for bcl-2 in vivo as an inhibitor of apoptosis in colorectal cancer.

The prevalence of BCL-2 protein immunocytochemical expression in colorectal cancers varies greatly from one study to another: Hilska M *et al*[29] 43%,; Saleh H *et al*[30] 51.9% . Those results were consistent with what we found in the current study.

[31]Goussia*et al*. investigated the expression of bcl-2 protein in a series of benign and malignant epithelial colorectal tumors. Investigated the usefulness of changes in Bcl-2 expression as prognostic factors in colorectal carcinoma. Those results were consistent with what we found in the current study.

The bcl-2 overexpression can be an early event in epithelial neoplasm carcinogenesis. These tumours frequently present distinct morphological stages, since benign hyperplasia, dysplasia, in situ carcinoma and finally invasive carcinoma[32], have indicated that the role of bcl-2 is, probably, more important in the initial development of colorectal tumours, keeping cells alive for late influence of others oncogenes, than in late phases of tumour progression.

[33]Yang *et al*. concluded that Bcl-2 expression appears at an early stage of the adenoma–carcinoma sequence and plays an important role in the early development of colon tumors.This conclusion was compatible with current study.

In colonic tissue, the physiological expression of bcl-2 protein is confined especially to the stem cells and at the base of crypts[34,35]. Evidently, the role of bcl-2 is to protect the stem cells and for the renewal and repair abilities of the epithelium from apoptosis[34].

There are studies which suggest that the majority of colorectal cancers express bcl-2[35], while in other studies this expression is observed in a lower proportion[9]. The fact that the BCL2 expression was not correlated with relevant clinicopathological parameters suggests that this oncogenic protein may play a role in the early stages of adenoma–carcinoma sequence, but probably its expression in established carcinomas has little significance[36].

Bcl-2 protein is a great inhibitor of apoptosis and its oncogenic activity is reflected in the prolonged cell survival . A mechanism with which bcl-2 protein protects the cell from apoptosis is not known – it is presumed that either there is a change in the mitochondrialfunc -tionor a change in regulation on the level of the cellularCa .++A high level of expression or the aberrant protein bcl-2 appears in different tumors[37].

In the present results, the positive results of BCL2-IHC detection ,where 45.2% of malignant colorectal tumors shows positive signals including 47.4 %,42.1 % and 10.5% in well differentiated carcinoma grade, moderately differentiated carcinoma, and poorly differentiated carcinoma ,respectively (Figure 4-18). Results

concerning the role of the bcl-2 protein in relation to prognostic parameters and survival of colorectal cancer are also conflicting.

A significant association was found between bcl-2 expression in our studied cases and tumor grade ; this was in agreement with Schwandnero *et al*.[38].

The role of bcl-2 in colorectal tumorigenesis is believed to be in the early stage of carcinogenesis. A decrease in the levels of bcl-2 can lead to cell death by apoptosis while it's over expression protects against programmed cell death[39].

Hegazy*et al*[39] showed the immunohistochemical evaluation of bcl-2 yields refined information on colorectal tumor biology with statistically significant relations with tumor grade.

[40]Bhatavdekar *et al*. had demonstrated that bcl-2 overexpression seems to be associated with advanced histological grade, resulting in a more aggressive tumor.

The relationships between p16 protein expression and colorectal adenocarcinoma have been investigated in a few studies[41-43]. The frequency of expression of p16 protein in colorectal adenocarcinoma reported varied from 17% to 99%, with the majority of studies showed p16 expression in more than two third of colorectal cancers[43].

[43]Alfred King *et al*. showed that p16 protein was expressed in 80% of the colorectal adenocarcinoma and approximately half (48%) of the tumors showed p16 overexpression.

The frequency of p16 protein expression noted in the study was similar to the frequency of p16 protein expression in colorectal mucinous adenocarcinoma[44]. These results are in an agreement with our results.

The overall high prevalence of p16 expression in colorectal adenocarcinoma implies that p16 alternations played an important role in the pathogenesis of this cancer[43].

[13]Caroline A.S *et al* displayed a positive staining reaction for the tumor suppressor gene p16 in 188 out of 200 cases (94%) cases while 12 (6%) specimens were negative of colorectal carcinoma.These results were consistent with our results.

High levels of pl6 would result in an inactive E2F (Rb-bound) and the arrest of the cell cycle at G,. This theory is supported by the observations that alterations of pl6 and Rb have an inverse correlation in some cell lines and that pl6 mRNA accumulates to a high level in cells lacking Rb function[45].

P16 gene encodes p16 protein that competes with cyclin D for binding to CDK4. This inhibits the ability of the cyclin D-CDK4 complex to phosphorylate Rb (retinoblastoma) protein, thus causing cell cycle arrest at late G1 phase. In recent years, the status of p16 alternations in cancer can be studied by immunohistochemistry. Strong p16 expression has been reported in many neoplasia[45].

A tumor suppressor gene, p16, was found to harbor promoter hyper methylation associated with the loss of protein expression in cancer cells, suggesting that p16 inactivation due to promoter methylation was important for colorectal tumorigenesis(18) .The reason for the presence of both methylated and un methylated p16 in some colon tumors is unclear. Authors speculated that this may reflect the heterogeneity of colon cancer.

P16 is a nucleoprotein, the presence of staining in both the nuclei and the cytoplasm supports the finding that p16 gene is overexpressed. The change in the subcellular location of the over-expressed nucleoprotein may account for the pathogenesis of colorectal adenocarcioma. It is apparent that the overexpression of p16 rather than the loss of its protein contributes to the pathogenetic mechanism of colorectal adenocarcinoma. Because of the frequent overexpression of p16 protein in colorectal adenocarcinoma, p16 overexpression may be use as a marker for colorectal adenocarcinoma for selected patients with histological diagnostic difficulty[43,46-47].

Our results indicate that the significance prevalence of BCL-2 as well as P16 - expression in colorectal carcinoma could point to an important contributing role of these molecular factors in the development and carcinogenesis of a subset of colorectal cancers.

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