ORIGINAL ARTICLE

Immunohistochemical Expression of BCL-2 in Colorectal Adenomas

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ABSTRACT

Background: The B-cell lymphoma-2 Gene (BCL-2) is a family of proteins, which control a critical step in commitment to apoptosis. Adenomatous polyps are small clump of cells lining or projecting above the surface of the colon; most of them are benign, harmless and noncancerous. However, over time, some of the polyps may develop into colon cancer.
Aims: To investigate the association between BCL-2 expression in colorectal adenomas, and to identify any association between BCL-2 marker and several histopathologic parameters (including tumor size, grade, site and type), in a sample of patients living in Erbil city, Northern part of Iraq.
Methods: A retrospective study done during the period between January 2017 till January 2018, on 101 formalin-fixed paraffin- embedded specimens of colorectal adenomatous polyps that were retrieved from Histopathology Laboratory at Rizgary Teaching Hospital and some private histopathology laboratories in Erbil city, Northern part of Iraq.
Results: Out of the 101cases, 85 of them showed positive BCL-2 expression. Thirty-four cases (33.67%) with high, 29 (28.72%) moderate, 22 (21.79%) low BCL-2 expression, and 16 (15.85%) showed no expression. Patients over 50 years showed highest number of expression(59.41%) compared to other age groups(P< 0.001). left side colon polyp predominate over right side (80, 79.21%), with majority of tubulo- villous type (P< 0.001).Nevertheless, no significant differences were found between gender, size of tumor, degree of dysplasia, and advanced adenoma
Conclusion: Over-expression of BCL-2 occurs early in colorectal adenoma polyps, which may act as one of important altered proteins expression leads to tumor cells proliferation and further progression to adenocarcinoma. The BCL-2 oncogene plays an important role in carcinogenesis by inhibiting cell death (apoptosis) that may enhance current cancer therapies and for future drug therapy discovery and targeting.
Keywords: Colorectal Adenoma, BCL-2, histopathologic features, Kurdistan.

INTRODUCTION

Colorectal cancer (CRC) is a common and lethal disease. CRC incidence and mortality rates vary markedly around the world. Globally, CRC is the third most commonly diagnosed cancer in males and the second in females[1,2]. CRC prevalence is expected to increase to more than 1.1 million of death and 2.2 million new cases by the year 2030[3]. More than 95% of colonic adenocarcinoma originate from polyps[4]. So the clinical significance of polyps arises from that fact. Conventional type adenomatous polyps can be classified as tubular, tubule-villous and villous adenomas. Villous adenomas are characterized by more than 75% villous component, where villous refers to finger-like or leaf-like epithelial projections. Tubulo-villous adenomas possess between 25% - 75% villous component, while tubular adenoma possess less than 25% villous components, the more the villous component in an adenoma, the more association with high grade dysplasia and increase risk of malignant transformation[5].

Genetics play a significant role in polyps development into cancer by inactivation of tumor suppressor genes such as adenomatous polyposis coli gene (APC), mismatch repair genes (MLH1) or loss of control of cell proliferation or cell death (apoptosis)[4].

The “Big Bang” model, which is based on the concept of punctuated evolution, implies that the majority of genomic alterations accumulate during the early stages of carcinogenesis, before the development of a big tumoral mass[6]. In this model, small colorectal polyps display different fates, with some growing and some regressing in size and others remaining stable in their size, the polyps growing are in time endowed with peculiar genetic/functional properties because they are “born to be bad” and possess multiple genetic abnormalities[7].

Apoptosis is a distinct form of cell death initiated by various physiological and pathological stimuli, that is morphologically characterized by cell shrinkage followed by formation of cell fragments, which are rapidly cleared by phagocytes that recognize “eat me” signals on the plasma membrane[8].

The BCL-2 oncoprotein (B-cell lymphocytic-leukaemia proto-oncogene) plays an important role in carcinogenesis by inhibiting cell death (apoptosis). It was initially discovered in follicular B cell lymphoma and subsequently in other malignant lesions[9]. Stimulation of apoptosis occurred upon exposure to harmful carcinogens or mutagenic agents, viral infections and ultraviolet radiations, commitment for cells to undergo apoptosis is triggered by extracellular or intracellular signals, which includes activation of caspase family involving two pathways. It is either the intrinsic pathway that mainly influences mitochondria permeability, which is also known as the mitochondria pathway, or the extrinsic pathway where involvement of direct interaction between the death ligand and its death receptor, or also referred to as the death-receptor mediated pathway[10].

Genomic changes leading to over expression of the anti-apoptotic proteins BCL-2, Bcl-xL and Mcl-1 are observed in a variety of neoplasms[11]. BCL-2 family has been discovered to play a key role in promoting or inhibiting intrinsic apoptotic pathway triggered by mitochondrial dysfunction. Therefore, the balance between pro- and anti-apoptotic members of this family can determine the cellular fate. Bax and BCL-2 are the major members of BCL-2 family whose potential roles in tumor progression and prognosis of different human malignancies have been of interest in various studies during the last decade. Bax promotes cell death through permeabilization of mitochondrial outer membrane in response to different cellular stresses. In contrast, BCL-2 prevents apoptosis by inhibiting the activity of Bax[12]. Being one of the earliest oncoproteins to be discovered, it is among the most notorious antiapoptotic proteins involved in malignant transformation. Owing to its anti-apoptotic properties, BCL-2 is over expressed in a variety of cancers, thereby allowing them to escape the built- in defenses against tumor formation, as well as to accumulate further mutations which cause progression to metastatic malignancy[13].
Inhibition of apoptosis is a fundamental element in carcinogenesis of colorectal cancer and also other human malignancies[14].

The objectives of the present study are to study the expression of BCL-2 in colorectal adenomas and to find the association between the expression of BCL-2 and demographic and pathological parameters.

MATERIALS AND METHODS

A retrospective study was conducted between the period from January 2017- January 2018, on 101 formalin-fixed paraffin-embedded blocks from
84 patients with adenomatous polyps that were retrieved from Histopathology Laboratory at Rizgary Teaching Hospital and some of the private histopathology labs in Erbil city, Kurdistan region, Northern part of Iraq. The inclusion criteria for the current study were electively resected colorectal adenomas based on histopathological results of endoscopically resected polyp specimens and no history of adenomatous polyposis coli or lynch syndrome. Three samples were excluded because of the insufficiency of materials.

The demographic data were obtained from records of the patients includes gender, age of the patients which includes(< 40, 40 – 50, and > 50 years), site of the biopsy which divided into right and left colonic polyps, size of the polyps which includes polyps less than 1 cm, between 1-2 cm and more than 2 cm, types of polyps includes (tubular, tubulo-villous and villous adenomas), grades of dysplasia in the polyps as low and high grade and lastly advanced adenomas that considers any adenomas which is more than 1cm in size and/or villous components and/or high grade dysplasia

Each polyp was analyzed individually, even when many polyps were resected from the same patient. A separate analysis was done on a sub-set data of 40 adenomas from17 patients harbor more than one polyp.

The study was approved by the ethic committee at College of pharmacy – Hawler Medical University (HMU-PH-EC No:191/05/99) .

All blocks were examined and the one which represented the best (no necrosis, no hemorrhage) was selected and new sections were made and stained by Hematoxylin and Eosin (H&E) for histological evaluation and were reviewed by a two independent experienced gastrointestinal pathologists and then the tissue samples submitted for immune staining for evaluation of BCL-2.

The tissue cuts were prepared in 4 µm thick and placed on special salinized slides. Then the samples were deparaffinized and put into a buffer solution for 20 min at 95-99°C, cooled in room temperature for 20 min. The method used was Dako Cytomation EnVision + System - horseradish peroxidase (HRP), which is a two-level IHC staining method. Monoclonal Mouse Anti- Human BCL2 Oncoprotein Clone 124 Code M0887.

The endogenous peroxidase activity was inhibited by incubation of slides inside peroxidase inhibitor for 10 min. The slides were incubated inside the primary diluted antibody for 30 min and then on labeled polymers for another 30 min. After staining, the slices were put in 3,3-diaminobenzidine (DAB) + substrate chromogen which detects the antigen in brown color. Lastly, the stained samples were reviewed under a light microscope and labeled cells were counted in both pathological and normal tissues for 100 cells in the microscopic field.

The scoring was performed, brown cytoplasmic staining of infiltrating lymphocytes serves as internal positive control while negative control was obtained by replacing the primary antibody with distilled water. The evaluation was conducted using a semi-quantitative scoring method by determining the proportion of positive cells over the total number of cells. Positive cells were determined by counting 1000 cells. All significantly stained cells were considered positive and divided by 10 to acquire the percentage. At least 10 HPFs were measured for each case for scoring. For BCL-2 expression, the density (was measured by the percentage of stained cells) was scored as 0 (for negative staining); 1 (for less than 10%); 2 (10–50%); and 3 (more than 50%).The intensity of staining(was measured by qualitative measurement)were evaluated at the same time as 0 (negative); 1(weak); 2 (moderate); and 3 (strong.). Then the results of density and intensity combined and scored from 0 to 6. The range again categorized as
(0) for negative expression, 1 and 2 for low expression, and 3 and 4 for moderate expression, and 5 and 6 for high expression[15].

Statistical Analysis: Data have been collected from files of patients, and entered in the computer and then analyzed using GraphPad Software statistical package computer software. Contingency tables have been formed to figure out the association between demographic and pathological parameters and BCL-2 expression. Chi-square test was used to calculate the significant differences between studied groups. A probability value of less than 0.05 was considered statistically significant[16].

RESULTS

Eighty five polyps out of 101 (84.18%) expressed BCL-2, from this (33.67%) with high expression, (28.72%)with moderate expression, and (21.79%) had low expression, while 16 (15.85%) showed no expression (Table 1)(Figure 1.B,C,D,E).

In normal colonic tissue, BCL-2 staining pattern was restricted to the base of colonic glands indicating that basal epithelial cells of the normal colonic crypts uniformly express the BCL-2 protein (Figure 1.A).

Fifty-six (56.44%) out of 101 cases were male compared to 44 (43.56%) females. Patients over 50 years showed highest number of expression (59.41%) compared to other age groups(P< 0.001). Left side colon polyp predominate over right side (80, 79.21%), with majority of tubulo-villous type (P< 0.001). Nevertheless, no significant differences were found between gender, size of tumor, degree of dysplasia, and advanced adenoma (Table 1).

The associations between the expressions of BCL-2 with the demographic and clinico-pathological parameters are presented in Table 2. There were significant associations with age (P=0.026), and type of the polyp (P=0.018). In contrast, no associations were found between expressions of BCL-2 with gender, site, size of polyp, degree of dysplasia, and advanced adenoma.

Table 2 revealed that majority of male and female patients showed BCL-2 expression (50.5% versus 33.7%). Patients with > 50 years showed the highest percentages of expression constituting(44.5%). Left colon polyps showed higher expression (65.3%) than right colonic polyp (18.8%). The results also showed that the size of polyps didn't influence the percentages of BCL-2 relatively. Tubulo-villous adenomas showed highest expression compared to both tubular and villous adenomas being (57.4%) versus (19.8%),(6.9%) respectively. No marked differences were seen between grades of dysplasia but marginal differences in percentages of incidence of advanced adenoma cases were observed.

Results clarified that a sub-set data of 40 adenomas out of the 101 (39.6%) were seen in 17 patients, which revealed to have more than one polyp (Table 3). 12 out of 17 patients (70.59%) found to have two polyps and 5 out of 17 (29.41%) found to have more than two polyps. There were a significant differences (P<0.001) in relation to site, types and grade of the polyps.

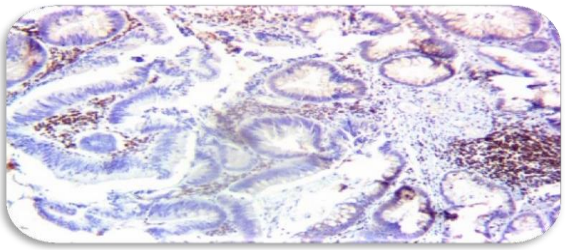
Left colon overpass right colon (92.5%). Moreover, Adenomas with high grade dysplasia were significantly higher (77.50%) than low grades dysplasia (22.50%). The number of patients with high grade dysplasia was significantly higher(70.59%) than low grades dysplasia(17.65%), while 2 (11.76%) patients had both grades.

Thirty polyps (75.0%) had villous component which was significantly higher among patients having more than one polyp (P< 0.001) while 12 (70.59%) of the 17 patients with more than one polyp found to have tubulo- villous type.

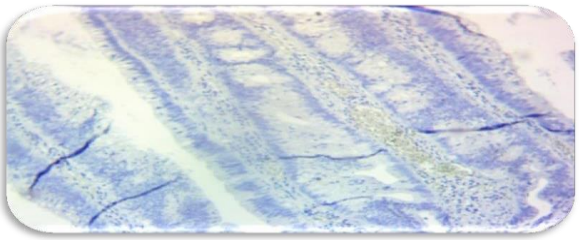
In contrast BCL-2 expression showed no significant difference in relation to, gender, age, size, and advanced adenoma.

Figure1. (A) Represent BCL-2 staining pattern was restricted to the base of colonic glands, (B) Negative expression, (C) low expression, (D)Moderate expression and (E) High BCL-2expression (X400 H&E counter staining).

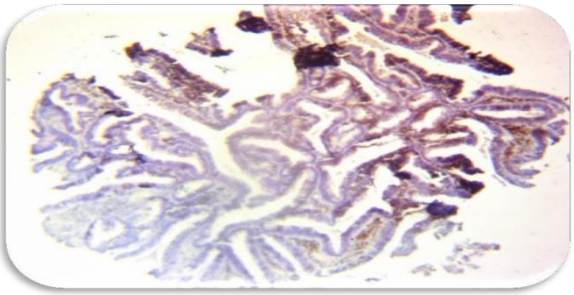
(A)



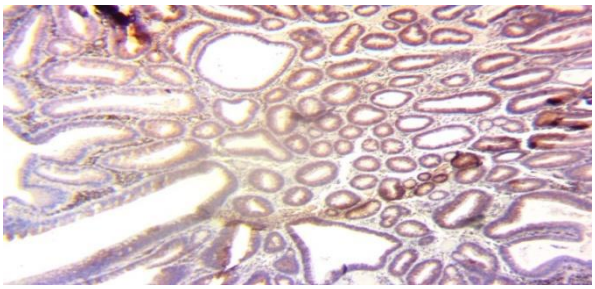
B)



C)



D)



E)

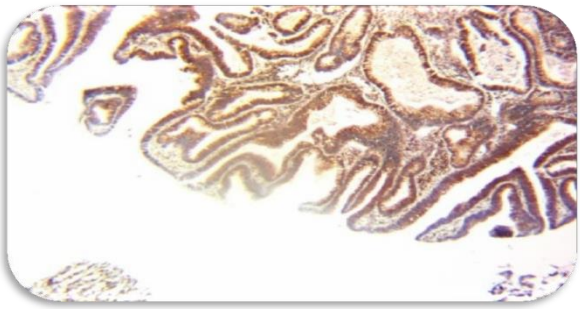


Table 1: BCL-2 expression, demographic and pathological parameters of the patients

Variables	Categories	No. of cases	(%)	P value
BCL-2 expression	High	34	33.67	0.060
	Moderate	29	28.72	
	Low	22	21.79	
	Negative	16	15.85	
Gender	Male	57	56.44	0.196
	Female	44	43.56	
Age	< 40	21	20.79	< 0.001
	40 – 50	20	19.80	
	> 50	60	59.41	
Site	Right colon	21	20.79	<0.001
	Left colon	80	79.21	
Size (cm)	<1	30	29.71	0.233
	1-2	42	41.58	
	>2	29	28.71	
Type of polyps	Tubular	21	20.79	<0.001
	Tubulo-villous	68	67.33	
	Villous	12	11.88	
Degree of dysplasia	Low grade dysplasia	54	53.47	0.424
	High grade dysplasia	47	46.53	
Advanced adenoma	No	61	60.40	0.045
	Yes	40	39.60	
Total		101		

Table 2: Association between BCL-2 and patients demographic and pathological parameters

Parameters	Variables	BCL 2 expression No. (%)		Total	P value
		Negative	Positive		
Gender	Male	6 (5.9)	51 (50.5)	57 (56.4)	0.095
	Female	10 (9.9)	34 (33.7)	44 (43.6)	
Age (years)	< 40	0 (0.0)	21 (20.8)	21 (20.8)	0.026
	40 – 50	1 (0.9)	19 (18.8)	20 (19.8)	
	> 50	15 (14.8)	45 (44.5)	60 (59.4)	
Site	Right colon	2(1.9)	19 (18.8)	21 (20.8)	0.373
	Left colon	14 (13.9)	66 (65.3)	80 (79.2)	
Size (cm)	< 1	5 (4.9)	25 (24.7)	30 (29.7)	0.938
	1-2	7 (6.9)	35 (34.6)	42 (41.6)	
	>2	4 (3.9)	25 (24.8)	29(28.7)	
Type of polyps	Tubular	1 (0.9)	20 (19.8)	21 (20.8)	0.018
	Tubulo-villous	10 (9.9)	58 (57.4)	68 (67.3)	
	Villous	5 (4.9)	7 (6.9)	12 (11.9)	
Degree of dysplasia	Low grade	7 (6.9)	47 (46.5)	54 (53.5)	0.396
	High grade	9 (8.9)	38 (37.6)	47 (46.5)	
Advanced adenoma	No	7 (6.9)	54 (53.5)	61	0.138
	Yes	9 (8.9)	31 (30.7)	40	
Total		16 (15.8)	85 (84.2)	101	

Table 3. Clinicopathological parameters for 40 cases of 17 patients with more than one polyp.

Variables	Categories	No. of cases (%)	P value	n(%)	P Value
BCL-2expression	High	13 (32.5)	0.060	4 (23.5)	0.321
	Moderate	15 (37.5)		4 (23.5)	
	Low	8 (20)		2 (11.8)	
	Negative	4 (10)		-	
	Mixed grades	-		7 (41.2)	
Gender	Male	20 (50)	> 0.05	9 (52.9)	> 0.05
	Female	20 (50)		8 (47.1)	
Age	< 40	11 (27.5)	0.231	5 (29.4)	0.601
	40 – 50	10 (25)		4 (23.5)	
	> 50	19 (47.5)		8 (47.1)	
Site	Right colon	2 (5)	< 0.001	1 (5.9)	< 0.001
	Left colon	37 (92.5)		15(88.2)	
	Both sites	1 (2.5)		1 (5.9)	

Size (cm)	<1	16 (40)	0.583	9 (52.9)	0.135
	1-2	13 (32.5)		4 (23.5)	
	>2	11 (27.5)		4 (23.5)	
Type of polyps	Tubular	5 (12.5)	< 0.001	1 (5.9)	< 0.001
	Tubulo-Villous	5 (12,5)		12(70.6)	
	Villous	30 (75)		1 (5.9)	
	Both polyps	-		3 (17.6)	
Degree of dysplasia	Low grade dysplasia	9 (22.5)	< 0.001	3(17.6)	< 0.001
	High grade dysplasia	31 (77.5)		12(70.6)	
	Both grades	- (0.0)		2 (11.8)	
Advanced adenoma	No	16 (40)	0.205	4 (23.5)	0.513
	Yes	24 60)		7 (41.2)	
	Both	-		6 (35.3)	
Total		40		17	

DISCUSSION

To determine the importance of BCL-2 in established colorectal adenomas we evaluated BCL-2 expression. This analysis revealed increased BCL-2 expression in transformed glands compared with normal tissue. The basal epithelial cells of the normal colonic crypts uniformly express the BCL-2 protein. The immunolocalization of BCL-2in the stem cells at the base of the crypt suggests that it protects stem cells from apoptosis[17].

The finding of a high level of homogenous diffuse in BCL-2 expression in adenomas in contrast to non-neoplastic cells, suggests that abnormal BCL-2 gene activation as an early event in colorectal tumorogenesis[17].
The results of this study revealed that 85/101(84.18%) polyps expressed BCL-2 with 63 of them expressed moderate to high expression.

These results are similar to results of Qasim *et al* who found high BCL-2 expression in 84.84% of cases he studied[18], while Watson *et al*[19] found 63.2% ,whereas Saneei and Razi [9] found BCL-2 expression in 92% of adenoma. In non-neoplastic mucosa, BCL-2 was expressed in the crypt cells only whilst the more differentiated surface epithelial cells lacked any demonstrable BCL-2 [20].

The homeostasis of the rapidly renewing intestinal epithelium is ensured by few stem cells present at the level of the base of intestinal crypts. Various experimental evidence suggests that colorectal cancers may derive from the malignant transformation of intestinal stem cells or of intestinal cells that acquire stem cell properties following malignant transformation. Colon cancer stem cells seem to be involved in tumor chemo resistance, radio resistance and relapse. Stem cell overpopulation not only initiates colon tumorigenesis, but also drives tumor growth [21].
It is worth to mention that Kaklamanis *et al.*[20] found BCL-2 staining pattern was consistently higher in adenomas than in carcinomas (78.6%) adenomas versus (51.9%) carcinomas, the same results were stated by Goussia *et al*.[22] thus BCL-2 expression is an early event in colorectal tumors.

In the current work, BCL-2 expression in colonic adenoma was higher in male gender, older age group (> 50years), left side colonic polyp, tubulo- villous type, and polyp with low grade dysplasia, which is nearly similar to result of Qasim *et al.*[18].
Our study revealed that BCL-2 expression is increased with increase in size of the polyp where it was 59.3% of polyps with size < 1cm and 1-2 cm, and only 24.8% in polyps of >2 cm ,this compatible with a study done by Saneei and Razi [9], where they found more BCL-2 expression in polyps (<1cm), the same result was also confirmed by Watson et al [19], who found Expression of BCL-2 was more extensive and intense in < 5 mm polyps, also Saneei and Razi [9], found a significant correlation between size of adenoma and BCL-2 expression, which suggested that BCL-2 may play an important role in the early stages of the adenoma-carcinoma sequence, down-regulation of BCL-2 is associated with the risk of malignant transformation for colorectal adenoma[23].

Several studies on the role of BCL-2 in colorectal carcinogenesis observed higher rates of BCL-2 expression in adenoma than carcinoma[9,24]. Altered apoptotic signaling begins in a benign adenomatous polyp, which develops into an advanced adenoma with high-grade dysplasia and then progresses to an invasive cancer, suggesting that BCL-2 might be linked to the early stage of CRC development[25]. It was suggested that over expression of the BCL-2 correlated with the transition between hyperplastic epithelium to adenomas[26]. Although the incidence of expression was significantly higher in adenomas for BCL-2,macroscopic morphology of colorectal adenomas is determined by the apoptosis not by proliferation, and high apoptosis found in depressed adenomas implies their low net growth[27]. The diffuse expression of BCL-2 and a superficial distribution of proliferating cells may contribute to the formation of a polypoid configuration in colorectal tumors. Thus, differences in both BCL-2 expression and apoptosis may play an important role in the morphogenesis of colorectal neoplasia[28].

Our study revealed that the BCL-2 protein is normally expressed along the crypts of normal colonic epithelium proportionate to the stem cell compartment where the apoptosis rate is low. The highest BCL-2expression is observed at the base and the lowest at the tip of the crypts. Continuous event of p53 mutation along the progression of CRC carcinogenesis is one of the factors affecting the BCL-2 expression. This scenario explained the decrease in apoptosis at the late stage of colorectal cancer and highlights the role of BCL-2 in the early stage of CRC. In addition, elevated expression of BCL-2 and deficiency in Bax might cause apoptosis-resistance in colon adenocarcinomas[29].

One of the keys to apoptosis regulation is the anti-apoptotic (BCL-2) which controls the release of pro-apoptotic factors. Various cancer types and malignancies

including CRC have been linked to the abnormal expression of BCL-2. Dysregulation of colonic epithelial cell apoptosis by abnormal expression of BCL-2 might leads to colorectal carcinogenesis[29].

Other study suggested a specific role of BCL-2 in stem-like cells in adenomas. A similar pattern of BCL-2 distribution was seen in human adenomas. It is speculated that BCL-2 inhibition might promote cell death in established adenomas[30].

In this context it should be mentioned that apoptotic signaling pathway is central to conserve a balance between cell death and survival and in keeping genome stability. As a rule, it is thought that the equilibrium between the rates of cell growth and apoptosis sustains intestinal epithelial homeostasis, and this stability is disturbed during cancer expansion[31, 32]. This types of apoptosis is under the control of proteins from the BCL-2 family. The protein BCL-2 is a key inhibitor of apoptosis, and its aberrant expression has been demonstrated in a wide range of solid tumors, including colorectal cancer. In non-pathological colon mucosa a high level of BCL-2 has been found at the level of base cells corresponding to progenitor cells[33]. Targeting BCL-2 anti-apoptotic complexes and pathways in cancer is a productive drug discovery and development field[34]. Apoptosis inhibition contributes to the survival and proliferation of tumors and plays an important role to current therapy resistance. Targeting apoptosis is therefore very promising for the development of new agents that may enhance current cancer therapies[35].

CONCLUSION

Over expression of BCL-2 occurs early in Colorectal adenoma polyps, which may act as one of important altered proteins expression leads to tumor cells proliferation and further progression to adenocarcinoma. The BCL-2 oncogene plays an important role in carcinogenesis by inhibiting cell death (apoptosis) that may enhance current cancer therapies and for future drug therapy discovery and targeting.

Competing interests: The authors declare that they have no competing interests.

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