The Expression of IGF- Binding Protein-related Protein 1 (IGFBP- rP1) In Colorectal Carcinoma (CRC) Among Diabetic and Non-Diabetic Patients

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Abstract
Insulin-like growth factor (IGF)-binding protein-related protein 1 (IGFBP-rP1) is a member of the Insulin Growth Factors (IGF) axis. IGFs plays role in glucose metabolism, promoting cell migration, mitogens, antiapoptotic and cell-survival factor. Type 2 Diabetes Mellitus or hyperinsulinemia state is hypothesized to promote colorectal carcinogenesis directly or indirectly by increasing the insulin-like growth factor-1 (IGFBP-rP1). This study was conducted to determine the expression and the association of IGFBP-rP1 protein in patients with colorectal carcinoma (CRC) with and without Diabetes Mellitus Type 2 (DM2). A total of 111 cases of CRC with or without clinically confirmed DM2 in Hospital Universiti Sains Malaysia Kelantan from year 2000 to 2010 were included. Results showed a significant expression of IGFBP-rP1 among CRC cases with (69%) and without DM2 (42%). Univariate analysis showed significant association of IGFBP-rP1 with DM2 (p<0.05), stage of the tumour (p=0.005) and tumour local invasion (p=0.037). These results were further strengthened using multiple logistic regression analysis. There was an increased expression of IGFBP-rP1 in colorectal cancer patients with diabetes mellitus type II. This study supports theory that chronic hyperinsulinemia indirectly promotes colorectal carcinogenesis via IGFBP-rP1, which might play an important role in the initiation and promotion of the cancer.
Keywords: *IGFBP-rP1, Colorectal Carcinoma, Diabetes Mellitus*

Introduction

Diabetes and cancer are common diseases with great impact on health worldwide. Type 2 diabetes is related with many types of epithelial cancers [1-3]. High associations are seen with liver[4], endometrium[5] and pancreatic cancer[6]. Reasonably high associations are seen in colorectal cancer[7], breast[8] and bladder[9]. Diabetes and cancer share many similar risk factors for cancer development [2,10]. Unfortunately, potential biologic links between the two diseases are not fully understood. Understanding the risk factors for CRC may provide strategies to prevent the development of the cancer [11,12]. Obesity and diabetes are strongly

associated [13,14]. Abdominal obesity is a known risk factor associated with hyperinsulinemia [15]. Epidemiologic studies support a relationship between insulin and colorectal carcinogenesis [16,17]. Insulin is a recognized growth factor that promotes proliferation of colon cancer cells in vitro and colonic tumours in experimental animals [18,19]. Chronic hyperinsulinemia may indirectly promote colorectal carcinogenesis by inducing concentrations of circulating IGF-1 and IGFBPs[20]. The IGF system is a complex molecular network that includes two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR), six high-affinity-binding proteins (IGFBP-1-IGFBP-6) and several binding-protein proteases [12,21]. IGF-I, IGF-II and the IGFBPs occur in high concentrations in the circulation and are readily measured. Understanding the IGF system could lead to improvement in treatment strategies[22]. Most cancer cells express insulin and IGF-I receptors; the A isoform was most commonly expressed. The A receptor isoform can stimulate insulin-mediated mitogenesis, even in cells deficient in IGF-I receptors [23]. The insulin receptor is also capable of stimulating cancer cell proliferation and metastasis. Most glucose uptakes in cancer cells is high, independent of insulin binding to its receptor [10].

Most type 2 diabetics are in a state of chronic hyperinsulinaemia. Hyperinsulinemia promotes carcinogenesis indirectly through its effects on IGF-I[10]. Insulin reduces the hepatic production of IGF binding protein (IGFBP)-1[24,25] and possibly IGFBP-2[26] with resultant increase in the levels of circulating free, bioactive IGF-I. IGF-I has more potent mitogenic and anti-apoptotic activities than insulin[27] and could act as a growth stimulus in preneoplastic and neoplastic cells that express insulin, IGF-I, and hybrid receptors[28]. Human tumours commonly over-express these receptors, and many cancer cell lines have been shown to be responsive to the mitogenic action of physiological concentrations of IGF-I. In addition to the direct effects of insulin, type 2 diabetes and/or the related obesity might enhance other pathways resulting in malignant progression. Adipose tissue is an active endocrine organ producing free fatty acids, interleukin-6 (IL-6), monocyte chemo-attractant protein, plasminogen activator inhibitor-1 (PAI-1), adiponectin, leptin, and tumor necrosis factor-α[29]. Each of these factors might play an etiologic role in regulating malignant transformation or cancer progression.

Kelantan, one of the states in Malaysia is ranked highest in prevalence of diabetes in the country in which the overall national prevalence is 8.3%[15]. The overall prevalence of diabetes mellitus in Kelantan in early 60s was not known, however for Malaysia was 0.65%[30]. By 2030, WHO estimates that would be 2.48 million diabetics in Malaysia, a jump of 164% from 0.94 million in 2002[31]. The trend of prevalence of overweight/obesity is also rising in Kelantan. In a study conducted in 2005[32] in Kota Bharu district in the state of Kelantan, the overall prevalence of overweight/obesity was 49.1%[32], much higher than the figure reported earlier in 1996[33]. With such a high prevalence of diabetes and obesity in Kelantan, a high incidence of colorectal carcinoma is expected[34]. A preliminary study from this state [35] noted a strong correlation between metabolic diseases with CRC.

The aim of this study is to determine the expression and association of IGFBP-rP1 protein in colorectal carcinoma cases of those with and without Diabetes Type 2 among Kelantan patients.

Materials and methods

This is a case control study comprising of cases histologically confirmed cases of colorectal carcinoma with or without clinically confirmed DM2 in Hospital Universiti Sains Malaysia (HUSM) Kelantan. The primary specimens were surgically excised colectomy samples of which paraffin embedded tissue blocks were archived in Department of Pathology of the same hospital. The specimens were received from year 2000 to 2010. Cases with inadequate clinical history or sections taken from endoscopic biopsies were excluded.

Immunohistochemistry (IHC)

The paraffin blocks were sections into 3 to 5 micron thickness and subjected to immunohistochemical stains using the standard method. Tissue sections for IGFBP-rP1 were placed into a beaker TRIS-EDTA pH 9 (retrieval solution) and were treated in the pressure cooker (full pressure) for 3 minutes (1x). The slides were cooled slowly in running water +/- 20 min. Sections were then rinsed in distilled water and Tris Buffer Saline (TBS). The sections were soaked into 0.3% hydrogen peroxide (H_2O_2) for 5 minutes to neutralize the endogeneous peroxidase activity, and then rinsed in distilled water. They were incubated overnight with a 1:200 dilution of goat polyclonal antibody against human IGFBP-rP1 [obtained from Labome catalog BAF1334]. IGFBP-rP1 primary antibody at 1:100 dilutions was applied to the sections and incubated for 10 minutes at room temperature. The sections were then washed once with TBS. Yellow drops from Link Antibody was applied to the section and incubated for 10 minutes. Sections were rinsed with buffer solution, applied Red drops of Streptavidin reagent and incubated for 10 minutes at room temperature. Then the sections were rinsed once with TBS. 3, 3'-diaminobenzidine was applied to the sections and incubated for 5 minutes at room temperature. The sections were then washed in running water twice, then counter-stained with 10 seconds dip in haematoxylin followed by bluing in the ammonia water. They were then washed in running water for 3-5 minutes before commencement of dehydrating procedure. The sections were immersed in alcohol with gradually increase in concentrations, from 50% to 80% and 95% (2 changes) and then into absolute alcohol (2 changes). Once ready, the sections were immersed in 3 changes of xylene, and finally mounted with DPX.

The immunohistochemistry stained sections were examined using light microscope (Olympus CX31, Japan). The scoring system used was based on published study of Shao L [36], where it was divided into 2 categories; 'low' when less than 50% cells are stained and 'high' when more than 50% tumour cells are stained by the antibody. For analysis 'low' score is considered negative. The sections were first evaluated by first author [AAMZ] and confirmed by senior pathologist, third author [NHO].

Statistical Analysis

The chi-square test was used to compare the immunoreactivity of IGFBP-rP1 expression among CRC cases with or without Type 2 DM. The association was determined using multiple logistic regression tests. All calculations were performed by using SPSS version 18.0, P-value < 0.05 was taken as statistically significant.

Results

A total of 111 colectomy specimens for colorectal cancers were recruited into the study. The mean age of the patients was 58.20 (13.34) years, the youngest was 18 and the oldest 86 years old. Etnic Malays was the majority followed by Chinese and Indian and male:female ratio was 1:1.1. Demographics data was presented in Table 1.

Table 1: The demographic and clinical features of Colorectal carcinoma patients (n=111)

Variable	n	%
Age		
≤30	3	2.7
>30	108	97.3
Ethnic		
Malay	92	82.9
Non-Malay	19	17.1
Gender		
Male	53	47.7
Female	58	52.3
Hypertension		
Yes	24	21.6
No	58	52.3
Unknown	29	26.1
CRC Differentiation		
Poor	4	3.6
Moderate	104	93.7
Well	3	2.7
Tumour Size		
<20	8	7.8
>20	94	92.2
Number of Positive Lymph Nodes		
<3	90	81.1
4-6	11	9.9
7-9	7	6.3
>9	3	2.7

Key: CRC: Colorectal carcinoma

Majority (109/111; 98.2%) of the patients in this study were beyond Duke stage B (Figure 1).
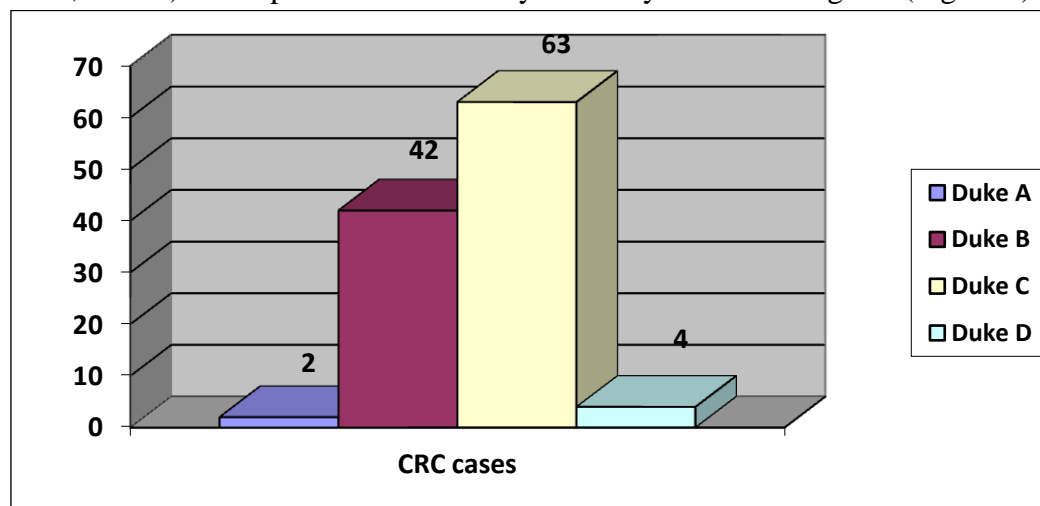

Figure 1: The Stage of Colorectal Cancer Diagnosed (n = 111)

Key:

Duke A: the cancer is only affecting the innermost lining of the colon or rectum or slightly growing into the muscle layer

Duke B: the cancer has grown through the muscle layer of the colon or rectum

Duke C: the cancer has spread to at least one lymph node in the area

Dukes D: advanced bowel cancer

From the total of 111 cases, 83 (74.8%) the diabetes status of the patients were known.   35/83 (42.2%) had diabetes mellitus Type 2 [DM2] (Figure 2).
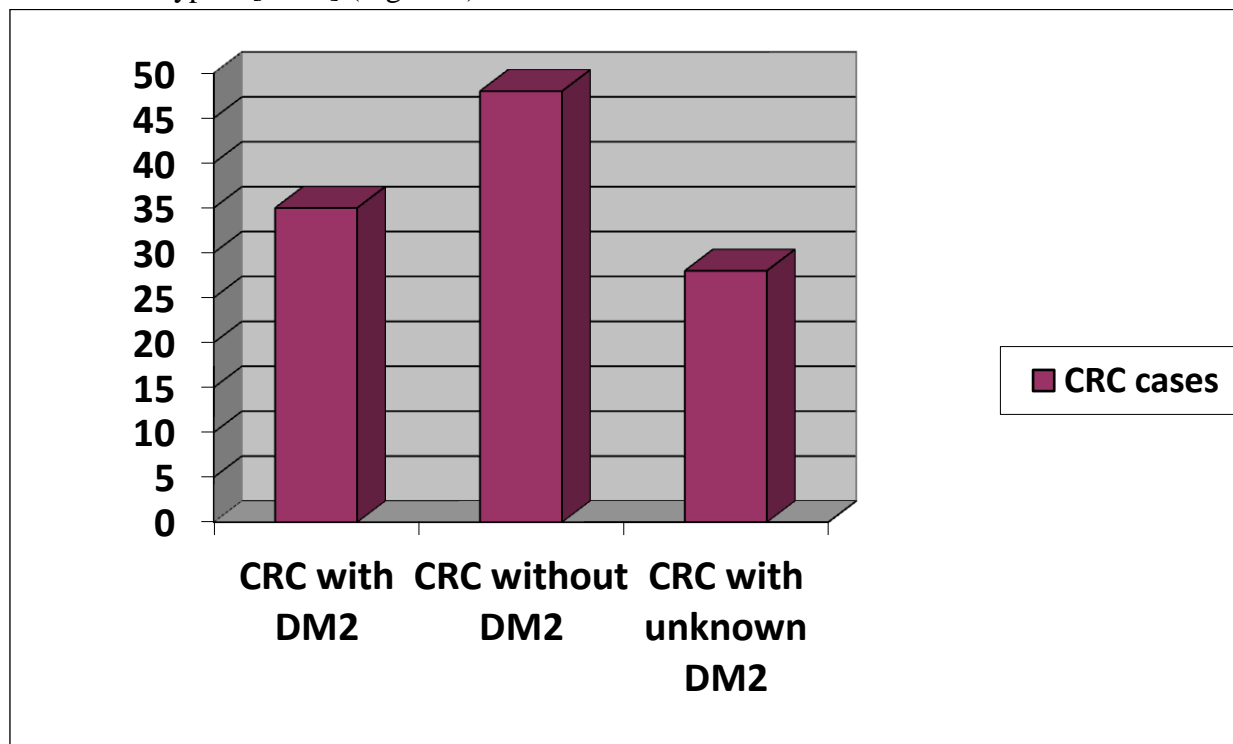

Figure 2: Graph showing the proportion of CRC cases with DM 2 status.

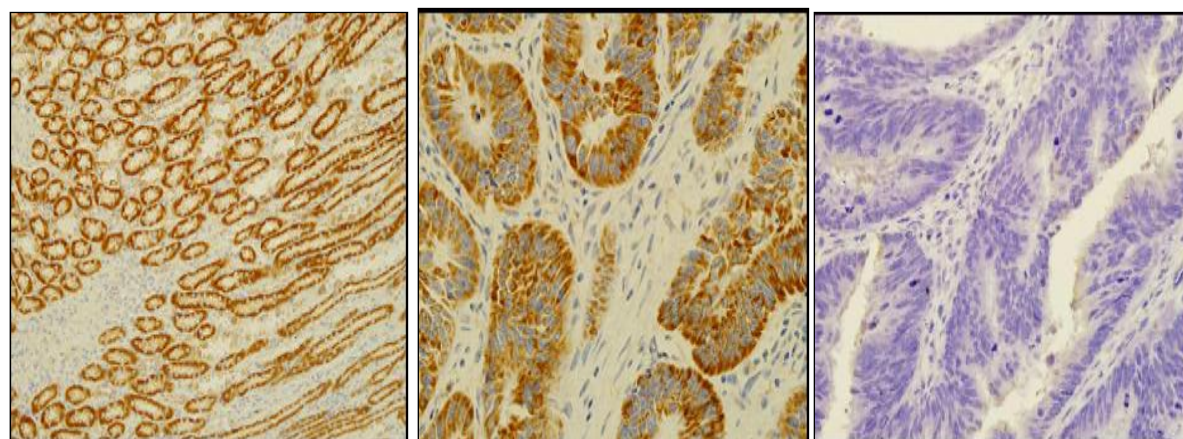Key:

CRC=Colorectal carcinoma; DM2= Diabetes Type 2

Positive expression of IGFBP-rP1 was seen in 42/83 (50.6%) of CRC cases with known DM Type 2. Of the 42 cases which expressed the protein, only 18 (18/42: 42.9%) cases were diabetic patients (Table 2).

Table 2: Immunohistochemistry (IGFBP-rP1) staining results according to colorectal carcinoma cases with or without DM2 (n=83)

Variables	Result for IGFBP-rP1 Staining	
	Positive (n)	Negative (n)
CRC with DM 2 Yes	18 (21.6%)	8 (9.6%)
No	24 (28.9%)	33 (39.8%)

Key: DM2: Diabetes Mellitus type 2. CRC: Colorectal carcinoma. IGFBP-rP1: Insulin Growth Factor Binding Protein related protein

The example of positive and negative expression of IGFBP-rP1 is shown in Figure 3a, b, c.

| a)Positive control: Normal rat kidney- histological section [supplied by vendor Labome catalog BAF1334] x200 magnification. | b)Histological section of positive expression of IGFBP-rP1 protein in Colorectal carcinoma X400 magnification. | c)Histological section of Colorectal carcinoma showing negative IGFBP-rP1 expression X 400 magnification. |

Figure 3a, b, c: Immunohistochemistry of colorectal carcinoma showing IGFBP-rP1 antibody staining.

There is a significant difference in the expression of IGFBP-rP1 among CRC cases with and without DM2. The over-expression of IGFBP-rP1 staining was observed in (18/26; 69%) of CRC cases with DM2, while only (24/57; 42%) in CRC cases without DM2. The univariate analysis showed significant association of this protein (IGFBP-rP1) with DM2 ($p<0.05$), the stage of the cancer ($p=0.005$) and local invasion of the tumour ($p=0.037$).

Multiple logistic regression test was done in order to establish the positive association in univariate analysis; DM2 ($p=0.019$; adjusted OR=3.50, 95% CI 1.23-9.97) and the cancer stage ($p=0.005$; adjusted OR=0.25, 95%CI 0.10-0.66) was statistically significant.

The age of the patients was positively associated with hypertension ($p=0.015$), tumour stage ($p<0.001$), number of lymph nodes present ($p<0.0001$) and number of lymph node with metastatic deposits ($p=0.007$). The number of lymph nodes present also was positively associated with DM 2 ($p=0.017$) and hypertension ($p=0.002$).

Table 3: The relationship between over expression of IGFBP-rP1 in tumour cells and clinicopathological variables

Variables	Result for IGFBP-rP1 Staining		Crude or (95% CI)	p-value
	+ve	-ve		
Age (years)			1	0.022[+]
Less than 30	18	8	3.09 (1.16, 8.28)	
More than 30	24	33		
Gender			1	0.591
Female	34	36	0.73 (0.23, 2.32)	
Male	8	6		
Ethnic			1	0.903
Malay	23	23	1.06 (0.44, 2.51)	
Non Malay	19	18		
Hypertension			1	0.881

Yes	27	27	1.07 (0.43, 2.64)	
No	15	14		
Diabetes Mellitus			1	0.005[+]
Yes	24	11	0.28 (0.11, 0.69)	
No	18	30		
Site of Cancer			1	0.202
Distal	6	55	0.51 (0.18, 1.40)	
Proximal	2	39		
Stage of the Cancer			1	0.367
Duke A & B	36	32	0.59 (0.19, 1.85)	
Duke C & D	6	9		
Tumor Size (mm)			1	0.385**
Less than 20	1	63	1.40 (0.59, 3.34)	
More than 20	25	45		
Positive Lymph Nodes			1	0.530
Less than 3	11	13	0.74 (0.28, 1.91)	
More than 4	31	27		

IGFBP-rP1: Insulin Growth Factor Binding Protein related protein.

[+]P-value < 0.05 is significant. *Analysis is done using Chi Square tests. ** Fisher's exact test is applied when expected frequency is less than 5

Discussion

Colorectal carcinoma is high in our population (state of Kelantan in Malaysia) and the incidence of diabetes is one of the highest in the country[34]. The link of diabetes and colorectal cancer is established [37,38]. While majority of patients with non-familial colorectal carcinoma is in the 60s[39],[40],[41] the patients in our series was slightly younger; 58.2(13.34), similar to a study from India [42]. Our hospital serves a predominantly ethnic Malay populations (98.0%); a rural setting in Malaysia. Though we noted majority of our cancer patients were among ethnic Malay 83% (92/111), the proportion of the cancer among ethnic Chinese was much higher 16/111(14%) as Chinese make up for less than 5% of the population served by the hospital. Goh Kl *et al* also noted a higher proportion of ethnic Chinese patients in their series of 3404 patients seen in urban setting in the same country[43]. In Malaysia ethnic Chinese is noted to have higher age standardized rate; 21/100,00 of this cancer compared to ethnic Malay;9.5/100,000[44]. The male to female ratio of our patients was 1:1.1 which correlates well with other findings [21]

though other studies showed male preponderance [23, 26-28].

Fifty percent of the CRC cases in this study showed a high expression of insulin like growth–binding protein–related protein 1 (IGFBP-rP1). 18 out of 42 cases were those of CRC with Type 2 DM. Our finding was similar with other studies [20, 35, and 36]. We noted that positive IGFBP-rP1 expression shows a significant association with the presence or absence of DM2 (p=0.022) and the stage of the tumour (p=0.005). Other clinicopathologic parameters showed no significant association with IGFBP-rP1 expression. We found an increased expression of IGFBP-rP1 in our colorectal cancer patients with DM2. This study was further strengthened by multiple logistic regression analysis whereby the IGFBP-rP1 expression was significantly correlated with DM2 status when adjusted for other parameters.

We observed patients with Diabetes mellitus Type 2 has a 3.5 times the odds (95% CI: 1.23 to 9.97, p-value 0.019) of expressing IGFBP-rP1 when adjusted for stage of the cancer. While patients with Duke Stage (A&B) and Duke (C&D) has a 0.25 times the odds (95% CI: 0.10 to 0.66, p-value

0.005) of expressing IGFBP-rP1 when adjusted for Diabetes mellitus status. This finding was almost similar to the study conducted by Shao et al.

We noted an increased expression of IGFBP-rP1 in colorectal cancer patients with diabetes mellitus type II. The IGFBP-rP1 was proposed to have the potential in predicting the risk of CRC development. This study supports the theory that chronic hyperinsulinemia may indirectly promotes colorectal carcinogenesis via IGF-1 bioavailability as a result of insulin-mediated changes in IGFBP concentrations.

Older patients are more of risk in getting colon cancer compared to younger age group unless they have strong family history of colon cancer. The incidence of most cancers increases with age [37]. In our study, we found that 108/111 (97.3%) patients are more than 30 years. Elderly patients are also more at risk to develop hypertension. We found colorectal cancer patients who were diabetic had significant association with hypertension, tumour stage, number of lymph nodes retrieved and number of lymph nodes with metastatic deposit.

The presence of Type 2 DM in CRC patient also showed a significant association with hypertension and number of lymph nodes with metastatic deposit. Diabetes and hypertension are grouped as metabolic diseases and they share similar risk factors. Among patients with hypertension, approximately half were insulin resistant. Insulin resistance might also increase blood pressure via reduced nitric oxide-mediated vasodilatation, increased salt sensitivity or plasma volume expansion [38].

Most (63%) of our CRC patients presented at Modified Duke's stage C. 42% of the cases were presented at Stage B and the remaining cases at Stage A and D respectively. This finding indicates that most of the patients were already in advanced stage at the time of diagnosis. Similar finding are seen in developed and developing countries [28-30].

Clinical presentations of CRC depend on the location of the tumour. Most common symptoms of distal colon were intestinal obstruction [21]. In our study, 65% of the CRC cases were located at distal colon (from transverse colon up to the rectum) and proximal colon accounted for 35% (caecum till ascending colon). Such was also noted in our earlier study [19] whereby 45% of the cases were located at recto-sigmoid colon.

Colorectal carcinoma spread locally by infiltrating adjacent structures. Haematogenous spread goes to the liver or lung and lymphatic spread to regional lymph nodes. Metastasis to numerous nodes, those close to the mesenteric margin, at great distance from the primary tumour, or in retrograde lymph nodes, have been associated with poor prognosis. However, prognostic value of identification of micrometastasis in lymph nodes by immunohistochemical or molecular techniques is still controversial [31, 32]. Though majority of our patients in this series present in advanced stage, majority had less than three lymph nodes involved by tumour (81.1%). Involvements of few lymph nodes have been reported to have good prognosis [31].

The grading was based on the percentage or proportion of glandular structures within the tumour. In our study, majority of the tumours were moderately differentiated (93.7%). These findings were similar with previous studies [27, 33, and 34]. Histological grade was an important prognostic factor [21]. However, the analysis for this parameter in our study was not significant.

In conclusion, there was an increased expression of IGFBP-rP1 in colorectal cancer patients with diabetes mellitus type II in our series. This study supports the theory that chronic hyperinsulinemia may indirectly promotes colorectal carcinogenesis via IGFBP-rP1, which might play an important role in the initiation and promotion of the cancer. In cases in which diabetes is not clinically detected, the expression of this protein in the cancer may give an indication that the patient could be diabetic.

Abbreviations:
CRC: Colorectal carcinoma
IGF: Insulin like growth factor
IGFBP-rP1: Insulin like growth factor – related protein 1
DM2: Diabetes mellitus Type 2

Conflict of interests: The authors declare that they have no conflict of interests.

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