

Detection of the differentially expressed gene IGF-binding protein-related protein-1 and analysis of its relationship to fasting glucose in Chinese colorectal cancer patients

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Abstract

IGF-binding protein-related protein-1 (IGFBP-rP1) is a member of the IGF axis. In our previous work, we separated cDNA fragments of IGFBP-rP1 from colonic adenocarcinoma and normal mucosa cDNA subtraction libraries. In this study, we compared the expression of IGFBP-rP1 by semi-quantitative RT-PCR and immunohistochemistry among colorectal cancer, adenoma, normal tissue adjacent to cancer site, and normal tissue. Associations between IGFBP-rP1 and plasma glucose were further explored. We found that the mRNA level of IGFBP-rP1 was highest in cancer, moderate in adenoma and tissue adjacent to the cancer site and lowest in normal tissue ($P < 0.05$). A significant difference was found in the immunoreactivity of IGFBP-rP1 between paired normal and cancer tissue ($P < 0.05$). Tumor samples with upregulated expression of IGFBP-rP1 in invading tumor cells showed an increased frequency of metastasis to the lymph node, an increased depth of infiltration and stronger staining of IGFBP-rP1 compared with other samples ($P < 0.05$). The fasting glucose level was significantly correlated with the staining of IGFBP-rP1 in cancer tissue (Spearman's $\rho = 0.4$, $P < 0.000$). Thus, we concluded overexpression of IGFBP-rP1 might play an important role in the initiation and promotion of colorectal cancer. IGFBP-rP1 expression may also be associated with fasting glucose level and the presence of diabetes mellitus.

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Introduction

Insulin-like growth factor (IGF)-binding protein-related protein 1 (IGFBP-rP1) is a member of the IGF axis, which is made up of a series of members including IGF-I and -II, IGF receptor-I and -II, IGFBPs and IGFBP proteases (Hwa *et al.* 1999). IGFs have numerous functions: they are potent mitogens, act as an anti-apoptotic survival factor, play a role in glucose metabolism, and promote cell migration (Pollak 2000, Wu *et al.* 2002). IGFBPs are divided into two categories according to their primary structure and affinity for IGFs – high-affinity IGFBPs (1–6) and low-affinity IGFBPs (7–15) (Hwa *et al.* 1999). IGFBP-rP1 is also identified as IGFBP-7, mac25 (meningioma-associated cDNA 25), PSF (prostatic-stimulating factor), AGM (angiomodulin) and

TAF (tumor-derived adhesion factor). It was the first identified low-affinity IGFBP (Baxter *et al.* 1998). Although IGFBP-rP1 has a relatively low affinity for IGFs, its affinity for insulin is very high and specific (Oh *et al.* 1996, Yamanaka *et al.* 1997).

The function of IGFBP-rP1 is complex and still largely unknown at present. It is widely distributed in normal tissues (Akaogi *et al.* 1996). This protein stimulates adhesion of vascular endothelial cells to plastic substrates and promotes the spread of rat liver cells through them (Akaogi *et al.* 1994). Upregulated expression of IGFBP-rP1 is observed in prostate adenocarcinomas (Degeorges *et al.* 1999), colon cancer (Umeda *et al.* 1998) and in cerebrospinal fluid of children with acute lymphoblastic leukemia (How *et al.* 1999). However, it

has also been demonstrated that downregulated expression of IGFBP-rP1 is a common phenomenon in tumors from prostate (Hwa *et al.* 1998, Sprenger *et al.* 1999, 2002), breast (Landberg *et al.* 2001) and liver (Komatsu *et al.* 2000) and in meningiomas (Murphy *et al.* 1993).

In our previous study, we separated the cDNA fragments of IGFBP-rP1 from colonic adenocarcinoma and normal mucosa cDNA subtraction libraries by suppressive subtractive hybridization. We found a higher expression of IGFBP-rP1 in colonic adenocarcinoma than in paired adenoma and normal tissue (Luo & Lai 2001). In this study, we demonstrated that upregulation of IGFBP-rP1 is common in colorectal cancer, especially in individuals with type II diabetes mellitus (DM). Based on these findings, we further explored the relationship between the expression of IGFBP-rP1 and plasma glucose.

Materials and methods

Tissue

Fresh colorectal cancer tissue was obtained from 76 colorectal cancer patients shortly after surgical resection. Paired fresh distant normal tissue was collected from 74 of 76 colorectal cancer patients; two of them had only tumor tissue available without distant normal tissue. Coincident adenoma samples were obtained for 25 of the colorectal cancer patients. Tissue adjacent to cancer sites was only collected in 43 patients. Concurrent cancer tissue, adenoma, tissue adjacent to the cancer site, and distant normal tissue were obtained in 12 patients. All samples were obtained from affiliated hospitals of Zhejiang University. Tissue adjacent to cancer sites was defined as grossly normal tissue located within 1–3 cm of a tumor. Distant normal tissue was grossly normal tissue located at 5 cm from the tumor. All samples were histologically confirmed by a pathologist. Fresh tissues were frozen in liquid nitrogen as soon as they were surgically removed and kept at -80°C . Portions of each specimen were fixed in 10% formaldehyde and embedded in paraffin with the exception of most adenomas (only five adenomas were embedded in paraffin) and all tissues adjacent to the cancer site due to their limited volume. Paraffin sections of the two normal tissues in patients without fresh normal tissues were obtained from the Department of Pathology of the affiliated hospitals of Zhejiang University.

Of the 76 samples, 37 were from tumors in the colon, while 39 were from tumors in the rectum. According to infiltration depth, four samples of tumor were limited to mucosa, 27 were limited to muscularis, and 35 infiltrated to serous or deeper. Twenty-eight of the 76 samples had metastases in surrounding lymph nodes. The disease archive of 67 patients was reviewed and six patients

were found to have comorbidity of type II DM. The criteria for the diagnosis of type II DM are listed as follow: fasting plasma glucose level ≥ 7.0 mmol/l or symptoms (polyuria, polydipsia, unexplained weight loss) plus casual plasma glucose levels ≥ 11.1 mmol/L or 2 h plasma glucose level ≥ 11.1 mmol/L during a 75 g oral glucose tolerance test.

Total RNA was prepared using Trizol reagent. The absorbance at 260 and 280 nm was measured regularly. The quality of total RNA was examined by running it on a 1% agarose/denaturing formaldehyde gel. Samples with a ratio of A_{260}/A_{280} at 1.8–2.0 and 28s/18s of approximately 2 were qualified for the next round.

Semi-quantitative RT-PCR

One microgram of total RNA was reverse transcribed by MuLV reverse transcriptase (MBI, Vilnius, Lithuania) with oligo (dT)₁₆ as a primer. Equal amounts of the reverse transcribed material were used as templates for amplification. Before application of RT-PCR in all samples, the amount of templates (40 ng for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), 150 ng for IGFBP-rP1 in a final volume of 25 μl) and cycles (24 for both G3PDH and IGFBP-rP1) were optimized to ensure that PCR products remained in the exponential phase of amplification. The primers for IGFBP-rP1 were sense (exon 2) 5'-cactgggtgccaggtgtact-3' and antisense (exon 4) 5'-ttggatgatggcactcatat-3'. The primers for G3PDH were 5'-accacagtccatgccatcac-3' and 5'-tccaccaccctgttctgtga-3'. PCR amplification was performed with 20 nM of each primer, 25 mM Mg^{2+} and 1 U Taq polymerase for 5 min at 95°C , followed by 24 cycles of denaturation (95°C , 30 s), annealing (59°C , 30 s) and extension (72°C , 30 s). Each RNA sample was analyzed in triplicate. Samples from the same patient were run in the same assay including the RT-PCR reaction and gel. The expected product was 240 bp for IGFBP-rP1 and 452 bp for G3PDH. The mRNA level of IGFBP-rP1 or G3PDH was assessed by measuring the density of the PCR-generated amplicon on the gel by a densitometric analysis. The expression of IGFBP-rP1 was calculated as a ratio of the level of G3PDH in the same tissue. The final expression level of IGFBP-rP1 was the average value of the three amplicons.

Immunohistochemistry of IGFBP-rP1

Paraffin-embedded sections (5 μm thick) were first dewaxed in xylene and rehydrated with a graded ethanol series. Endogenous peroxidase was quenched by incubation in 3% H_2O_2 for 10 min at room temperature. Non-specific binding was blocked by incubation in a 1:10 dilution of rabbit serum for 30 min at room temperature.

Then, sections were incubated at 4°C overnight with a 1:200 dilution of goat polyclonal antibody against human IGFBP-7 (C16, SC6064; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After several washes in PBS, the sections were incubated with a 1:200 dilution of biotinylated rabbit IgG at room temperature for 30 min. Then, the slides were incubated in a 1:200 dilution of rabbit horseradish peroxidase at room temperature for 20 min. The peroxidase activity was visualized by incubating in 0.06% 3-3'-diaminobenzidine-H₂O₂. The sections were finally counter stained with hematoxylin. PBS was used to replace primary antibody in the negative controls. Slides with positive staining of IGFBP-rP1 were further incubated with neutralized primary antibody (neutralized by SC 6064P; Santa Cruz) as negative control.

The staining of IGFBP-rP1 was divided into four grades (0–3) according to Landberg *et al.* (2001), representing lack of staining (0), mild staining (1), intermediate staining (2) and strong staining (3).

Plasma glucose

Five of six samples with comorbidity of type II DM showed intermediate–strong staining of IGFBP-rP1. Therefore, we reviewed the patients' disease archives to obtain the level of fasting plasma glucose before surgery for each patient. Disease archives were only available for 67 of the 76 patients.

Statistical methods

A one-way ANOVA and the Friedman test were applied when comparing the expression of IGFBP-rP1 in different tissues. The chi-square test was used to compare the immunoreactivity of IGFBP-rP1 among different stages of colorectal cancer. Associations between IGFBP-rP1 and fasting glucose were calculated using Spearman's rho two-tailed test. All calculations were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

The average age of patients was 57.9±13.9 years. Eleven (14.5%) normal tissues showed very low to undetected mRNA level of IGFBP-rP1, while variable mRNA levels were observed in adenomas and cancer samples. We observed a significant difference in the expression of IGFBP-rP1 among the four types of tissue (Table 1). In 12 samples with paired normal tissue, adenoma, tissue adjacent to cancer site and cancer sample, ten of them had at least a 1.5-fold or greater expression of IGFBP-rP1 in cancer as compared with normal tissue (Table 1).

Immunohistochemistry was performed on 76 tumor samples, 5 adenoma samples and 76 distant normal tissue

samples. IGFBP-rP1 is located in cytoplasm. Immunostaining of IGFBP-rP1 was detected in epithelial cells of 59.2% (45 of 76) normal tissues, 60.0% (3 of 5) adenomas and 85.5% (65 of 76) cancer tissues. Significant differences in positive rates were observed between tumor samples and normal tissue (chi-square=12.0, $P < 0.05$). Weak staining, intermediate staining, and strong staining were detected in 36, 22 and 7 of the tumor samples respectively (Fig. 1b–d). The mRNA level of IGFBP-rP1 was positively associated with its immunostaining level (correlation coefficient=0.29, $P = 0.03$). If histologically normal mucosa and/or dysplasia were present in the same section of tumor, an increasing staining of IGFBP-rP1 can be seen from the normal tissue and/or dysplasia to cancer (Fig. 1b–d). In 29% (22 of 76) samples, the cancer cells located in the invasive front of the cancer nest showed stronger staining of IGFBP-rP1 than those surrounding the lumen (Fig. 1e and f). Those samples had an increased frequency of metastasis to lymph node, an increased depth of infiltration and stronger staining of IGFBP-rP1 than other samples (Table 2). Five of the six samples with comorbidity of type II DM showed intermediate–strong staining of IGFBP-rP1; only one showed mild staining.

Fasting glucose

The staining of IGFBP-rP1 was divided into two categories here: low (including grades 0 and 1) and high (including grades 2 and 3). The level of fasting glucose before surgery in the group of high IGFBP-rP1 was significantly higher than that of low IGFBP-rP1 (Table 3). Six patients with type II DM received different medication before surgery, so the practical fasting plasma glucose level might be partially masked by the treatment. But after exclusion of these six samples, the difference in fasting plasma glucose level between the IGFBP-rP1 high-expression group and low-expression group was still significant (data not shown).

Discussion

We had previously reported that IGFBP-rP1 was differentially expressed between cancer tissue and normal tissue in a certain colon cancer patient. Increased expression was found in cancer tissue by virtual Northern blot and RNA dot-blot (Luo & Lai 2001). In the present study, we further strengthened the previous report concerning increased expression of IGFBP-rP1 in colorectal cancer. This is consistent with the report of Umeda *et al.* (1998). Tumor samples with upregulated expression of IGFBP-rP1 in invading tumor cells showed an increased frequency of metastasis to lymph nodes and an increased depth of infiltration. This finding

Table 1 The expression of IGFBP-rP1 in colorectal cancer, adenoma, tissue adjacent to the cancer site and paired normal tissue

No.	mRNA level of IGFBP-rP1				Immunostaining grade of IGFBP-rP1 in tumor
	Normal tissue	Adenoma	Tissue adjacent to tumor	Tumour	
1	0.64	NA	0.71	0.29	0
2	1.72	NA	0.20	1.10	0
3	0.81	1.73	NA	1.77	1
4	0.00	NA	0.00	1.47	0
5	0.14	NA	0.83	0.76	2
6	2.61	NA	NA	2.94	2
7	1.00	0.43	NA	0.64	1
8	0.30	NA	NA	0.89	0
9	0.18	0.29	0.00	0.54	1
10	0.02	NA	0.01	0.23	0
11	0.20	0.01	1.04	0.35	2
12	0.00	0.00	0.00	0.13	1
13	0.08	0.07	0.01	0.12	2
14	0.13	NA	0.03	0.19	1
15	0.20	0.19	NA	0.25	2
16	0.25	NA	0.19	0.69	1
17	0.19	0.26	NA	0.30	2
18	0.21	0.84	NA	0.63	1
19	1.62	1.92	1.57	3.26	1
20	0.00	NA	0.35	0.17	1
21	0.46	NA	0.45	0.64	0
22	0.35	NA	0.60	0.73	2
23	0.70	NA	0.43	0.40	1
24	0.00	NA	0.00	0.84	3
25	0.00	0.50	NA	0.69	1
26	0.00	0.29	NA	0.50	1
27	0.13	0.03	0.10	0.76	1
28	0.71	0.45	0.36	1.29	2
29	0.27	0.08	0.35	0.34	1
30	0.30	NA	0.35	0.59	2
31	0.13	NA	0.90	2.04	3
32	0.51	NA	NA	1.09	3
33	0.11	NA	0.01	0.30	1
34	0.27	0.39	0.10	0.46	1
35	1.04	1.14	NA	1.44	3
36	0.18	NA	NA	0.40	1
37	0.97	0.98	NA	0.75	1
38	0.57	NA	NA	1.55	1
39	0.50	NA	NA	1.05	2
40	0.20	NA	NA	0.20	0
41	0.30	NA	NA	0.44	0
42	0.49	0.56	NA	0.75	2
43	0.27	NA	NA	0.48	2
44	0.48	NA	NA	0.50	1
45	0.07	NA	0.06	0.01	1
46	0.05	NA	0.02	0.69	1
47	0.10	0.28	NA	0.38	1
48	0.21	NA	NA	0.33	1
49	0.00	NA	NA	0.18	1
50	0.03	NA	NA	0.13	3
51	0.24	NA	NA	0.46	1
52	0.08	NA	NA	0.44	1
53	1.00	1.73	1.28	1.96	2
54	1.33	NA	NA	2.14	2

Table 1 continued

No.	mRNA level of IGFBP-rP1				Immunostaining grade of IGFBP-rP1 in tumor
	Normal tissue	Adenoma	Tissue adjacent to tumor	Tumour	
55	0.00	NA	NA	0.80	2
56	0.00	0.30	NA	1.54	3
57	2.70	NA	3.30	1.80	2
58	0.04	NA	0.39	0.82	2
59	0.00	0.98	1.07	1.39	0
60	1.04	1.46	1.32	1.23	1
61	0.81	NA	0.95	0.96	0
62	0.32	NA	0.29	0.33	2
63	NA	NA	0.00	0.22	1
64	0.03	NA	0.45	0.19	1
65	0.38	NA	0.64	0.84	2
66	0.29	NA	NA	0.68	2
67	NA	NA	NA	0.10	1
68	0.10	NA	NA	0.13	1
69	NA	1.00	NA	1.22	2
70	0.38	NA	0.15	1.21	1
71	0.66	NA	0.35	1.31	1
72	0.01	NA	0.21	0.22	1
73	0.00	NA	0.00	0.35	0
74	0.39	NA	0.45	1.69	1
75	1.28	NA	0.51	0.61	2
76	7.21	NA	7.94	3.05	3

The difference in mRNA level of IGFBP-rP1 among the four groups is significant by the Kruskal–Wallis test (chi-square=25.4, $P = 0.001$). The Spearman's rho between mRNA level; and immunostaining grades in cancer tissues is 0.29 ($P = 0.03$). NA, not available.

complements that of Adachi *et al.* (2001). They found that high expression of IGFBP-rP1/AGM in invasive tumor cells is associated with poor prognosis. These results suggest that upregulation of IGFBP-rP1 may be related to the initiation and promotion of colorectal cancer. IGFBP-rP1 is an adhesive factor (Degeorges *et al.* 1999); its overexpression may result in easier anchorage of tumor cells to the endothelium or matrix, promoting tumor cells to metastasize.

The upregulation of IGFBP-rP1 may be caused by changes in the gene structure or abnormalities of upstream regulators. The same restriction fragment patterns have been demonstrated among normal breast cell lines and breast cancer cell lines which have down-regulated expression of IGFBP-rP1 (Swishelm *et al.* 1995). These results indicate that IGFBP-rP1 is not grossly rearranged or deleted in tumor lines. In mouse liver cancer, the downregulation of mac25 is shown to be caused by the hypermethylation of a NotI site 6 bp downstream of the putative translation initiation site (Komatsu *et al.* 2000). But in the sequence of the human mac25 gene, this NotI recognition sequence is absent.

Therefore, the relationship between upregulation of IGFBP-rP1 in colorectal cancer and gene structure change requires further validation.

IGFBP-rP1 is an autocrine/paracrine factor. Many factors, including cortisol (Pereira *et al.* 1999), retinoic acid (Swishelm *et al.* 1995), IGF-I (Hwa *et al.* 1998), transforming growth factor- β (Hwa *et al.* 1998), $1\alpha,25(\text{OH})_2\text{-D}_3$ (Kanemitsu *et al.* 2001) and parathyroid hormone (Pereira & Canalis 1999), have been demonstrated to be capable of enhancing the expression of IGFBP-rP1. These results suggest that IGFBP-rP1 expression change may be caused by abnormalities in upstream regulators.

The most probable regulator is IGF-I. IGF-I has been proven to upregulate the expression of IGFBP-rP1 in the prostate cancer cell line, P69 (Hwa *et al.* 1998). Also, IGFBP-rP1 can bind IGF-I with a specific affinity (Oh *et al.* 1996). It has been shown to stimulate fibroblasts to secrete prostaglandin E2 (PGE2) (Yamauchi *et al.* 1994). PGE2 can enhance IGF-I synthesis through a cAMP-dependent protein kinase A pathway. On the other hand, IGF-I has been demonstrated to potentiate interleukin- β .

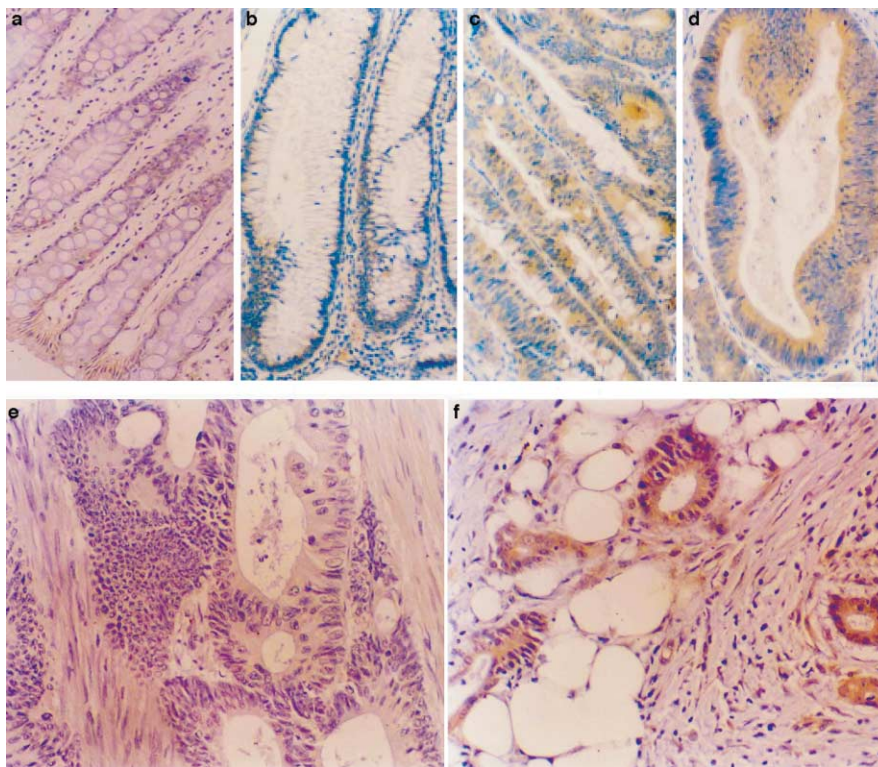


Figure 1 Immunostaining of IGFBP-rP1 in colon tissues $\times 400$. (a–d) Were from the same patient, (b–d) were from the same slide. (a) Negative staining in epithelium of distant normal tissue; (b) weak staining in mild dysplasia; (c) moderate staining in moderate dysplasia; (d) strong staining in cancer tissue. (e and f) Were from the same slide. (e) Negative staining of tumor cells which infiltrated to muscularis; (f) moderate staining of tumor cells infiltrating to fat tissues.

Table 2 The relationship between overexpression of IGFBP-rP1 in invading tumor cells and clinicopathological variables

Character	IGFBP-rP1 overexpression in invading tumor cells		P value
	Positive (22 cases)	Negative (54 cases)	
Age (years)	60.1 \pm 12.2	57 \pm 12.5	NS
Sex			
Male	11	33	NS
Female	11	21	
Site			
Colon	13	24	NS
Rectum	9	39	
Lymph node			
Positive	12	16	0.04 ¹
Negative	10	38	
Infiltration depth			
Mucosa	0	4	0.01 ¹
Muscularis	4	23	
Serous or deeper	18	27	
Staining of IGFBP-rP1			
0	1	10	0.04 ¹
1	9	27	
2	9	13	
3	3	4	

NS, no significance. ¹Chi-square test.

Table 3 The association between IGFBP-rP1 immunoreactivity and the level of fasting glucose

IGFBP-rP1	Case	Mean (mM)	S.D. (mM)
Low ¹	43	5.38	1.29
High ²	24	6.00	0.94

¹Including grade 0 and 1; ²including grade 2 and 3. One-way ANOVA: $F=4.23$, $P=0.04$; Spearman's $\rho=0.4$, $P<0.000$.

induced cyclooxygenase-2 expression in mesangial cells. This, in turn, leads to enhanced PGE2 (O'Byrne *et al.* 2000). Whether IGFBP-rP1 is involved in the PGE2 synthesis induced by IGF-I is not known. In summary, there is a complex network among IGF-I, IGFBP-rP1 and PGE2. IGF-I may be linked with IGFBP-rP1 by direct binding or by the PGE2 pathway indirectly. Additionally, IGF-I treatment has been shown to increase the incidence of colon cancer transplantation and the frequency of hepatic metastasis in mice. It stimulates angiogenesis in tumors. The neovascularization is found to be most frequent at the invasive front (Wu *et al.* 2002). The distribution pattern of new blood vessels in colon cancer is similar to that of IGFBP-rP1 staining. This coincidence may be a common characteristic belonging to infiltration- and metastasis-promoting genes, but we cannot exclude an inner association between them.

Another regulator candidate is insulin. Patients with type II DM usually have an elevated level of plasma insulin. We found that five among six samples with type II DM had an intermediate–strong expression of IGFBP-rP1. In streptozotocin-induced type I DM rats, the expression of PSF in renal vessels has been shown to be significantly decreased (Ono *et al.* 1998). These results suggest that insulin may play a role in regulating the expression of IGFBP-rP1. However, we cannot conclude that the overexpression of IGFBP-rP1 is caused by IGF-I and/or insulin because we have not detected the level of IGF-I or insulin.

We are surprised to find that most colorectal cancer patients with type II DM demonstrated relatively high expression of IGFBP-rP1 and the expression was significantly related to the level of fasting glucose. Hyperglycemia may be a risk factor for colorectal cancer. Populations with a level of fasting glucose at the top 25% show an increased risk of 1.8-fold of colorectal cancer than those at the lowest 25%. Populations with a level of plasma glucose and insulin 2 h after feeding at the top 25% show an increased risk of 2.4- and 2.0-fold respectively in comparison with those at the lowest 25% (Ma *et al.* 1999). Hyperglycemia may be linked to IGFBP-rP1 by enhancing the level of circulating IGF-I or insulin (Forbes *et al.* 1989). On the other hand, overexpression of

IGFBP-rP1 may block the binding of insulin to its receptor and further affect the metabolism of glucose. It is difficult to draw conclusions merely from the evidence from the disease archives.

Conclusion

As stated above, our findings conclude that overexpression of IGFBP-rP1 may be related to the initiation and promotion of colorectal cancer. Its overexpression in invading tumor cells may be linked to the increased infiltration potential. The expression of IGFBP-rP1 may be associated with the level of fasting glucose and the presence of type II DM. Although an upregulated expression of IGFBP-rP1 was observed in our study, we cannot exclude the possibility that IGFBP-rP1 is a tumor suppressor, because in many other studies IGFBP-rP1 plays an anti-proliferation role (Hwa *et al.* 1998, Sprenger *et al.* 1999, 2002, Landberg *et al.* 2001). Further study on IGFBP-rP1's exact role in colorectal carcinogenesis and its relationship with insulin, IGF-I and plasma glucose should be completed in order to further understand the role of IGFBP-rP1 and its relationship between colorectal cancer and type II DM.

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