Co–Expression and Relationship between IGF–Ⅱ mRNA and Bc l–2 Oncoprotein in Chinese Colorectal Adenocarcinomas

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Abstract **Objective** to investigate the relationship between the expression of IGF-Ⅱ, Bcl-2 and the invasion, metastases of colorectal adenocarcinomas and to study the clinical significance of their expressions. **Methods** Forty-eight paraffin embedded samples from colorectal adenocarcinomas were selected. IGF-Ⅱ mRNA was detected by in situ hybridization, the expression of Bcl-2, Proliferating cell nuclear antigen (PCNA) protein were detected immunohistochemically, and apoptosis was detected by TUNEL technique. Fifteen normal colorectal tissues were used as controls. The specimens with positive cell ratio≤ 30% were defined as negative. **Results** The expression of IGF-Ⅱ mRNA and Bcl-2 protein were significantly higher in colorectal adenocarcinomas (38.70%±7.80% and 30.97%±7.40%) than those in normal colorectal tissues(23.12%±4.07% and 12.69%±1.31%)(P<0.01) and were related to Dukes′stage and LN metastases, but were unrelated to patient age, gender, tumor site, tumor size and tumor differentiation. A negative correlation was observed between IGF-Ⅱ mRNA and Bcl-2 protein (P<0.05) and both of them related with Dukes′stages and metastases of tumor. A positive correlation between IGF-Ⅱ mRNA and PCNA, apoptosis, as well as a negative correlation between Bcl-2 and apoptosis were observed(P<0.01). There was no correlation between Bcl-2 and PCNA (P>0.05). The patients with IGF-Ⅱ mRNA(+) and Bcl-2(-) were regarded as the worst prognosis. **Conclusion** The overexpression of both IGF-Ⅱ and Bcl-2 in colorectal adenocarcinomas play an important role in the pathogenesis, progression, invasion and metastases of tumor. The detection of IGF-Ⅱ and Bcl-2 co-expressions would be helpful for the clinical adjuvant therapy to some extent.

Key words IGF-Ⅱ; Bcl-2; Colorectal adenocarcinomas; In situ hybridization

Insulin-like growth factor Ⅱ (IGF-Ⅱ) is a kind of cytokines that have strong effect of promoting cell mitosis and proliferation. B cell lymphoma 2 (bcl-2) gene is so far regarded as the most important gene that inhibits cell apoptosis. Overexpression of IGF-Ⅱ or bcl-2 has been reported in many types of tumours, including liver carcinoma, breast carcinoma, prostate cancer, colorectal carcinoma, smooth muscle tumours, liposarcoma and others, which was considered tightly connected with angiogenesis and progression of the tumours[1~6]. In this study, we first investigated the expression of IGF-Ⅱ mRNA, bcl-2 protein and the relationship between the expression and the invasion, metastases of colorectal adenocarcinomas. The aim of the study is to provide theoretical help for diagnosis and therapy of Chinese colorectal adenocarcinomas.

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MATERIALS AND METHODS

The paraffin embedded specimens obtained from 48 patients who underwent surgical treatment for colorectal adenocarcinomas between November 1, 2000 and July 31, 2001 in the department of oncology, Zhongnan Hospital, WuHan University, WuHan, P. R. China. All the patients did not accepted chemical therapy before operation and were testified by pathology. There were 27 males and 21 females with a mean age of 55 years (ranging from 28 years to 77 years). There were 9 colon carcinomas and 39 rectum carcinomas with 25 Dukes′ stage A, 6 Dukes′ stage B, 13 Dukes′ stage C and 4 Dukes′stage D. In addition, 15 normal colorectal tissues were used as controls. All the specimens were fixed with 10% formalin, embedded with paraffin and cut as 4μm thickness.

In Situ Hybridization of IGF–Ⅱ

The in situ hybridization is to detect the responding

gene sequence in situ tissue with specific DNA probe under the premise that which structure of chromosomes, cells and tissues are preserved integrally. The probes of IGF-Ⅱ labelled with biotin at the 5′end, whose sequence is 5'-GTGCTTCTCACCTTCGCCTTCGCCTC GTGCTGCATTG-3′, were synthesized in ShangHai Sangon Bioengineering Company, ShangHai, P. R. China. The sections were firstly deparaffinized in xylene and rehydrated. After endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide at 37℃, nonspecific binding was blocked. The sections were then dehydrated with 100% alcohol. When samples air dried, the sections were incubated about 20 hours at 45℃ with the probes at a concentration of 16μg/ml. The sections were then washed respectively with 2×SSC and 0.1×SSC buffered saline containing 0.1% SDS, and were incubated with Streptavidin-HRP for 30 minutes at 37℃. Finally, Diaminobenzidine(DAB) was used as a chromagen and the sections were counterstained with hematoxylin. The cells in which cytoplasm was stained brown yellow were defined as positive results under microscope.

Immunohistochemical Analysis of bcl-2 and PCNA

Serial paraffin sections of 48 tissues from the patients examined for in situ hybridization of IGF-Ⅱ were used for immunohistochemical staining of bcl-2 and PCNA. Immunohistochemical staining of bcl-2 and PCNA were performed according to the protocol of Streptavidin-Peroxidase Coiugated (S-P) Method Detection Kits (bcl-2 monoclonal antibody No.100/D5; PCNA monoclonal antibody No. PC10, Fuzhou MaiXin Biotechnology Company, FuZhou, P. R. China). In bcl-2 positive sections, the cytoplasm of cell was stained brown yellow, however in PCNA positive sections, the cell nuclei was stained brown yellow.

Analysis of Cell Apoptosis

Serial paraffin sections of 48 tissues from the patients examined for in situ hybridization of IGF-Ⅱ were used for detection of cell apoptosis. The apoptosis of cell was analyzed according to the guideline of the TUNEL Technique Apoptosis Detection Kit (Promega Company, America). In apoptosis positive sections, the cell nuclei was stained brown yellow.

Judgment of the Results

Five kens(×400)under optic microscope were selected in each section at random. The mean positive cell ratio of tumor of IGF-Ⅱ, bcl-2, PCNA, apoptosis were obtained by HPIAS2000 style analysis system (TongJi Qianping Photograph Engineering Company, WuHan, P. R. China). Referring to the standards of Kawamato and Alexandra, the specimens in which positive cell ratio of IGF-Ⅱ, bcl-2 ≤ 30% were defined as positive, while others were negative.

Statistical Analysis

Statistical Analysis was by Pearson method, student′s test or Chisquane test by means of SPSS10.0 software. For all statistical analyses, the value of $P<0.05$ was considered to be significant.

RESULTS

In Situ Hybridization of IGF-Ⅱ

The positive signals of hybridization of IGF-Ⅱ mRNA distributed mostly at cytoplasm were observed in all specimens (Fig.1). The expression of IGF-Ⅱ mRNA was significantly higher in colorectal adenocarcinoma (38.70%±7.80%) than that in normal colorectal tissues (23.12%±4.07%)($P<0.01$).

Immunohistochemical Staining of bcl-2 and PCNA

Positive staining of bcl-2 and PCNA were observed in all specimens. The expression of bcl-2 protein was mostly detected both in cytoplasm and cytomembrane (Fig.2), while the PCNA protein was mostly expressed in cell nuclei (Fig.3). The expression of bcl-2 protein and PCNA protein were significantly higher in colorectal cancer (30.79%±7.40% and 84.30%±4.51%) than those in normal colorectal tissues (12.69%±1.31% and 53.20%±7.50%)($P<0.01$).

Analysis of Cell Apoptosis

The stained apoptotic cell could be observed in all specimens. In some positive cell, the apoptosis body could be seen (Fig.4). The apoptotic cell ratio was significantly higher in colorectal cancer (26.88%±8.60%) than that in normal colorectal tissues (5.30%±1.30%) ($P<0.01$).

Relationship between the Positive Cell Ratio of IGF-Ⅱ, bcl-2 and Clinical Pathological Characteristics

The expression of IGF-Ⅱ mRNA and bcl-2 protein were related to tumor Dukes′ stage and lymph node metastases ($P<0.01$), but were unrelated to patient age, gender, tumor site, tumor size or tumor tissues differentiation(Table 1).

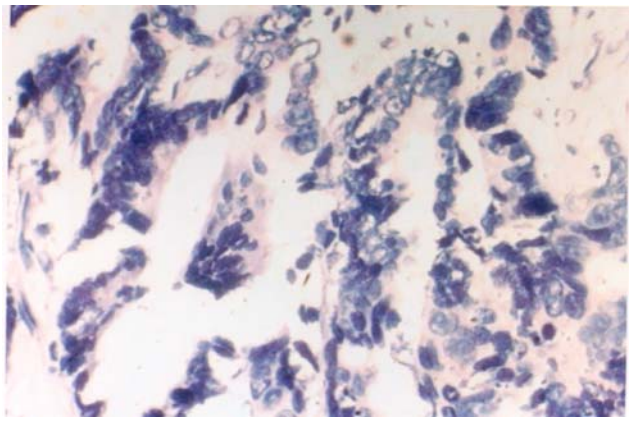

Fig.1 In situ hybridization by anti-sense IGF-Ⅱ biotinylated labeled probe. Expression of mRNA was observed as an intense brown yellow signal(×400)

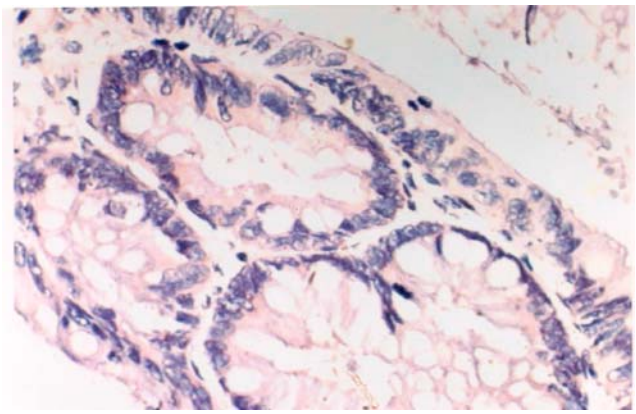

Fig.2 Immunohistochemical staining with anti-bcl-2 antibody in colorectal adenocarcinomas In bcl-2 positive sectious, the cytoplasms and cytomembrane of the cells was stained(×400)

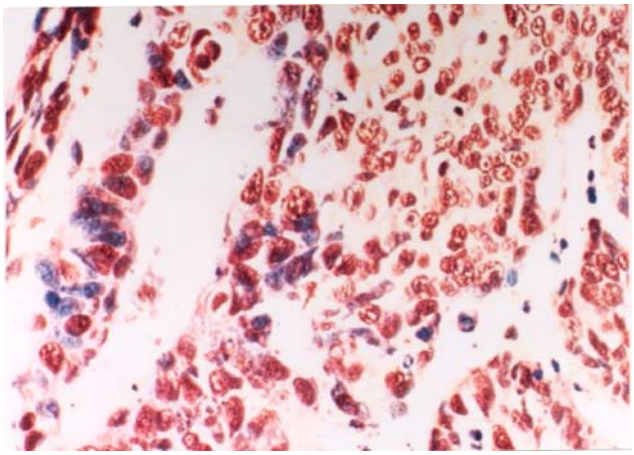

Fig.3 Immunohistochemical stainnig with anti-PCNA antibody in colorectal adenocarcinomas In PCNA positive sections, the nuclei of the cells was stained as brown yellow(×400)

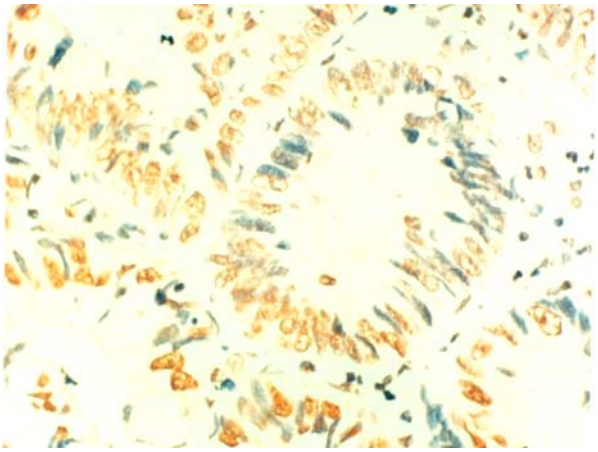

Fig.4 The apoptosis situation was observed by TUNEL method in colorectal adenocarcinomas. In apoptosis positive sections, the nuclei of the cells was stained as brown yellow(×400)

Correlation Among Expression of IGF-Ⅱ, Bcl-2, PCNA and Apoptosis

A negative correlation was observed between IGF-Ⅱ mRNA and bcl-2 protein (P<0.05). A positive correlation between IGF-Ⅱ mRNA and PCNA, apoptosis, as well as a negative correlation between bcl-2 and apoptosis were observed(P<0.01). There was no correlation between bcl-2 and PCNA (P>0.05). In addition, a positive correlation between PCNA and apoptosis was also observed (P<0.01) (Table 2).

Co-expression of IGF-Ⅱ and bcl-2

IGF-Ⅱ positive specimens (I+) were divided into two teams, Dukes′stage A,B and Dukes′stage C,D. Then the difference was observed between the two teams according to the expression of bcl-2 protein. The outcome of statistical analysis by Chisquane test was χ^2=12.75 (P<0.005) (Table 3).

DISCUSSION

IGF-Ⅱ gene is located at Chromosome No.11. IGF-Ⅱ protein is a single-chain polypeptide which molecular weight is 7.5 KDa. As a growth factor, IGF-Ⅱ has strong ability to promote cell proliferation[9]. It is reported that IGF-Ⅱ plays an important role in fetal growth.

Table 1 Comparison between the Positive Cell Ratio of IGF-Ⅱ, Bcl-2 and Clinical Pathological Characteristics in Chinese Colorectal Adenocarcinomas

	n	IGF-Ⅱ (%)	P value	Bcl-2(%)	P value
Age					
≥ 50y	27	37.72±7.40	0.336	32.74±7.80	0.06
<50y	21	39.95±8.40		28.70±6.30	
gender					
Male	27	39.56±8.50	0.394	29.82±7.80	0.228
Female	21	37.58±7.00		32.45±6.70	
Dukes′stage					
A	25	32.81±3.99	0.001*	34.79±5.70	0.969*
B	6	41.79±3.78	0.246**	34.89±5.50	0.001**
C	13	44.29±4.32	0.009***	24.84±4.80	0.159***
D	4	52.69±6.45		21.10±1.81	
Site					
Rectum	39	38.91±7.95	0.703	31.09±7.56	0.808
Colon	9	37.78±7.99		30.42±7.11	
Size					
>3×3cm²	23	40.49±7.29	0.132	30.46±6.89	0.650
≤ 3×3cm²	25	37.05±8.19		1.44±7.96	
Differentiation					
Well	7	36.40±5.33	0.409△	31.20±5.12	0.953△
Moderately	36	39.17±8.40	0.876△△	31.38±7.60	0.331△△
Poorly	5	38.54±7.60		27.72±9.10	
LN metastases					
Positive	15	45.24±5.32	0.000	24.33±4.77	0.000
Negative	33	5.73±7.06		33.99±6.37	

* Stage A compare with Stage B; **Stage B compare with Stage C; *** Stage C compare with Stage D
△ Well Differentiation compare with Moderately Differentiation,
△△ Moderately Differentiation compare with Poorly Differentiation

Table 2 Correlation between IGF-Ⅱ, Bcl-2, PCNA and Apoptosis Expression in 48 Cases of Chinese Colorectal Adenocarcinoma (The Number in the TABLE Stands for Correlation Coefficient r)

	IGF-Ⅱ	Bcl-2	PCNA	Apoptosis
IGF-Ⅱ	——	-0.432*	0.522△	0.655△
Bcl-2	-0.432*	——	-0.341○	-0.550△
PCNA	0.522△	-0.341○	——	0.502△
Apoptosis	0.655△	-0.550△	0.502△	——

* $P<0.05$ △ $P<0.01$ ○ $P>0.05$

Table 3 Relation between Co-expression of IGF-Ⅱ, Bcl-2 and Dukes′stage

	A and B	C and D
I⁺B⁺	18	4
I⁺B⁻	5	14

+: positive -: negative

After birth, however, IGF-Ⅱ levels decrease, and only a few tissues (for example liver and nerves) express IGF-Ⅱ in the adult[10]. Bcl-2 proto-oncogene is regarded as the most important gene that inhibits cell apoptosis recently. Bcl-2 protein which molecular weight is 26 KDa exists in all kinds of tissues in normal condition with the physiological function of adjusting the velocity of cell apoptosis and keeping the relative balance of cell number[11,12]. There are two important aspects in adjusting cell number, proliferation and apoptosis, which act and im-

pact each other. Overexpression of IGF-Ⅱ or/and bcl-2 can disturb the balance and result in the increasing of cell number, especially the premature and older cells. The tumor will be more easily happened if the cells are attacked by many chemical, physical, biological risk factors. In many researches, overexpressions of IGF-Ⅱ, bcl-2 were observed in many kinds of tumors and were related to the angiogenesis and progression of tumors. But the correlation between IGF-Ⅱ, bcl-2 in Chinese colorectal adenocarcinomas is not clear.

In our study, expression of IGF-Ⅱ mRNA, bcl-2 protein was significantly higher in colorectal adenocarcinomas than in normal colorectal tissues (P<0.01) which was testified by many scholars[13,14]. Compared with clinical pathological characteristics, the expression of them was related to tumor Dukes′ stage and lymph node metastases, but was unrelated to patient age, gender, tumor site, size and differentiation. Overexpression of IGF-Ⅱ mRNA was much stronger in Dukes′ stage D than that in Dukes′ stage A and a significant correlation between high IGF-Ⅱ mRNA level and tumor lymph node metastases was observed. On the contrary, expression of bcl-2 protein was the lowest in terminal carcinoma. The lower level of bcl-2 protein helps to metastases. The interesting results was that significant negative correlation between IGF-Ⅱ and bcl-2 was observed. Because the tumor cell apoptosis ratio was significantly higher in terminal period than that in early period[15], as the related genes of cell proliferation and apoptosis, IGF-Ⅱ and bcl-2 were compared respectively to PCNA, cell apoptosis. A positive correlation between IGF-Ⅱ mRNA and PCNA, apoptosis, as well as a negative correlation between bcl-2 and apoptosis were observed. No correlation was found between bcl-2 and PCNA. That means the function of IGF-Ⅱ was performed by promoting cell proliferation and apoptosis, but the function of bcl-2 was performed only by inhibiting cell apoptosis. Our results suggested that in early period of tumor, IGF-Ⅱ promoted cell proliferation, but bcl-2 inhibited cell apoptosis and partly counteracted the increasing of cell apoptosis caused by IGF-Ⅱ. These resulted in the increasing drastically in cell number. So bcl-2 could play a more important role in tumor angiogenesis than IGF-Ⅱ relatively. Accompany with the development of tumor, expression of IGF-Ⅱ was stronger and stronger. At the same time, expression of bcl-2 was weaker and weaker (higher than in normal tissues). Not only did the cell proliferation, but also the cell apoptosis increased. Accordingly a new balance was built between cell proliferation and apoptosis at an active basis. The reason that increasing cell apoptosis in terminal tumor was considered by some researchers that the tumor cells were purified by rendering the apoptosis of non tumor cells which was generated in the process of overproliferation to take place. On the other hand, the apoptosis of "weaker" tumor cells enabled the invasive tumor cells to grow constantly, providing a good physical base for tumor metastases[16]. In conclusion, co-overexpression of IGF-Ⅱ mRNA and bcl-2 protein promoted commonly the angiogenesis, progression, invasion and metastases of colorectal adenocarcinomas. IGF-Ⅱ and bcl-2 play important role in adjudging prognosis[17,18]. But the internal mechanism of interaction of IGF-Ⅱ and bcl-2 is not clear, and need further study.

In addition, the relationship was analyzed between bcl-2 and Dukes′ stage when expression of IGF-Ⅱ was positive. In our study, the specimens in which IGF-Ⅱ is positive and bcl-2 is negative signified the worst prognosis. The detection of co-expression of IGF-Ⅱ, bcl-2 may give some helps for making therapeutic project and adjudging prognosis.

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