Clinical Significance of Tissue Expression of Interleukin-6 in Colorectal Carcinoma

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Abstract. *Background: Although serum interleukin-6 (IL-6) has been associated with the development of colorectal cancer, IL-6 expression in cancer tissues has never been elucidated. The role of the tissue expression of IL-6 in colorectal cancer was investigated to identify any prognostic significance. Materials and Methods: One hundred and sixty consecutive patients, whose primary lesions had been resected, were studied. Immunoreactivity of IL-6 in cancerous tissue was measured by immunohistochemical staining; the relationships between the positive expression of IL-6 and both clinicopathological factors and survival were evaluated. Results: Seventy-four specimens expressed IL-6, and this expression correlated with an elevated serum carcinoembryonic antigen level, lymph node metastasis, venous invasion and advanced stage ($p<0.05$). IL-6 expression correlated with poor survival ($p<0.05$) and was an independent predictor of prognosis. Conclusion: Because tissue expression of IL-6 correlates with aggressive colorectal cancer behavior, it may be a useful predictor of prognosis.*

Interleukin-6 (IL-6) is a pleiotropic cytokine involved in the regulation of immune reactions. The ability of some cancer cell lines to produce and secret IL-6 into culture supernatants has been demonstrated by several researchers (1-5). Secreted IL-6 binds to a membrane receptor (IL-6R) composed of ligand-binding (gp 80) and signal-transducing subunits (gp 130) (6), causing up-regulation of functions involved in carcinogenesis (7). Specifically, IL-6/ IL-6R complexes initiate homodimerization of gp 130, activate a cytoplasmic tyrosine kinase, and trigger signaling cascades through the JAK/STAT, Ras/MAPK and PI3-K/ AKT (8) pathways; these actions regulate inflammatory reactions, immune responses, and several other pathophysiological processes of malignancy including cell growth and survival, differentiation, cell mobility and angiogenesis (9-12). IL-6/IL-6R autocrine activity is implicated in the development and progression of cancers including cervical cancer, prostate cancer and multiple myeloma (9-14).

In addition, associations of increased serum IL-6 with poor prognosis of cancers of organs including the stomach, prostate, ovary and breast have been reported (1, 15-18). Several studies indicated that serum IL-6 is involved in metastasis and tumor progression of colorectal cancer (19-23). We have observed that the serum IL-6 level correlates with the survival of colorectal cancer patients, but not as an independent prognostic factor (24, 25). Serum IL-6 is produced by several kinds of cells, including macrophages, monocytes, fibroblasts and cancer cells. Kinoshita *et al*. reported that 60% of colorectal cancer specimens overexpressed IL-6 (21). It is not known whether overexpression of IL-6 in colorectal cancer reflects the biological characteristics of the tumor. The current study investigated the correlations between the tissue IL-6 level and patient survival and determined whether tissue IL-6 was an independent prognostic factor for colorectal cancer.

Materials and Methods

Patients. The study group consisted of 160 consecutive patients (age range, 24-96 years; 105 men, 55 women) who had undergone resection for localized colorectal cancer from April 1997, to December 2003, at Hsin-Chu Tao-Yuan General Hospital, Taiwan. The protocol was approved by the hospital review board, and informed consent was obtained preoperatively. Patients with inflammatory disease, infection and bowel obstruction or perforation were excluded. Tumors were located in the cecum or ascending colon in 43 patients (26.9%), transverse colon in 4 patients (2.5%), descending colon in 4 patients (2.5%), sigmoid colon in 38 patients (23.8%) and rectum in 71 patients (44.4%). All primary cancers were excised. Under TNM classification, 1 patient had stage I disease, 69 patients had stage II, 68 patients had stage III and 21 patients had stage IV. All patients with lymph node or

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Key Words: Interleukin-6, tissue expression, colorectal cancer, prognosis.

other metastasis were treated postoperatively with adjuvant chemotherapy. Conventional chemotherapy, including 5-fluorouracil and leucovorin, was given to stage III patients. Patients with synchronous or metachronous metastasis received second-line chemotherapy with oxaliplatin and 5-fluorouracil. All patients were followed until March 2005, or death. The median postoperative follow-up was 37.5 months.

Colorectal carcinoma specimens and uninvolved mucosa were obtained during surgery. Hematoxylin and eosin-stained tumor sections were examined. Histological grade, size, site, carcinoembryonic antigen (CEA) level, tumor invasion (venous, lymphatic, perineural), lymph node status, pathological stage and survival time were all recorded. All protein expression assessments for this study were carried out without knowledge of the pathological or surgical data. The tumor expression of IL-6 and IL-6R were evaluated in 160 and 159 cases by an immunohistochemical method. Serum IL-6 concentration was measured for 88 cases using an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Inc., Woburn, MA, USA). The cut-off point was selected as 12 pg/mL (24).

Immunohistochemical staining. Goat polyclonal antibodies to IL-6 and rabbit polyclonal antibodies to IL-6R were purchased from Chemicon Corp. (Temecula, CA, USA). Formalin-fixed paraffin-embedded archival tissue was sectioned to 3-μm thickness at room temperature. Serial sections were de-waxed in xylene and dehydrated serially in graded ethanol. Endogenous peroxide activity was quenched by incubating the specimen for 20 minutes in 3% hydrogen peroxide followed by a 5-min incubation with blocking reagent to prevent nonspecific staining. The tissue sections were reacted with primary antisera for IL-6 (1/100) and IL-6R (1/200) at room temperature for 2 h. The slides were washed in phosphate-buffered saline containing 0.1% Tween 20 for 15 min, changing the solution three times, followed by incubation with biotinylated secondary antibody for 15 min and streptavidin-biotin complex reagent for 15 min, all at room temperature. The antigen-antibody reaction was visualized using 0.02% 3, 3'-diaminobenzidine as a chromogen, and the sections were counterstained with hematoxylin. Negative controls were created by omitting the primary antibody.

Quantitative analysis of immunoreactivity. The expression of IL-6 and IL-6R in normal mucosa and carcinoma was evaluated pair-wise. To avoid intratumoral heterogeneity, the deepest invasive sites were selected for evaluation. The percentage of positively-stained tumor cells was evaluated for each section after counting 1,000 cells per high-power field. Expression of immunoreactive IL-6 was classified as positive, when >30% of cells showed cytoplasmic staining; expression of immunoreactive IL-6R was classified as positive, when >30% of cells showed staining of the cytoplasm and cell membrane. Two independent investigators, blinded to the nature of the specimens, evaluated the slides.

Statistical methods. The results are shown as mean±standard deviation (SD). Fisher's exact test, Chi-square and two-sample *t*-tests were used to compare clinicopathological data. Survival curves were made using the Kaplan-Meier method, and the log-rank test was used to analyze the differences. Multivariate analyses were performed using the Cox proportional hazard model. $P \leq 0.05$ was regarded as statistically significant.

Results

IL-6 immunoreactivity was observed intensely in the cytoplasm of colorectal carcinoma cells; it was detectable in 74 out of 160 patients with colorectal carcinoma (46.3%, Figure 1). IL-6R immunoreactivity was observed in the cytoplasm and plasma membrane of colorectal carcinoma cells; it was detectable in 56 out of 160 patients with colorectal carcinoma (35%, Figure 2). In contrast, IL-6 and IL-6R immunoreactivity was detected in less than 10% of the normal mucosa specimens (12/160 and 15/160). The clinicopathological factors *versus* tissue expression of IL-6 are shown in Table I. Elevated CEA levels were significantly more common in the IL-6(+) group than in the IL-6(–) group ($p<0.05$). Advanced TNM stage was significantly more common in the IL-6(+) group than in the IL-6(–) group ($p<0.05$). IL-6 overexpression was also associated with lymph node metastasis and vascular invasion by the tumor ($p<0.05$). However, the tissue expression of IL-6 did not correlate with the serum expression of IL-6 ($p=0.122$). Table II shows the relationship between IL-6R overexpression and clinicopathological data. No significant correlation was found between IL-6R and these factors. Table III summarizes the relation between IL-6 and IL-6R expression. IL-6R immunoreactivity was expressed to a greater degree in tumors with IL-6 immunoreactivity than in tumors without IL-6 immunoreactivity ($p<0.01$).

The difference in 5-year overall survival between patients with and without tissue expression of IL-6 was significantly different ($p<0.01$, Figure 3), though it was not significantly different in patients with and without IL-6R expression ($p>0.05$). Multivariate analysis with logistic regression for the 5-year overall survival rate showed that stage, CEA level and tissue expression of IL-6 were significant independent predictors of poor prognosis (Table IV).

Discussion

Mechanisms leading to high serum concentrations of IL-6 in cancer patients include CEA-induced IL-6 production by Kuppfer cells, tumor-associated macrophages, or tumor cells themselves (19, 26, 27). IL-6 appears to enhance tumorigenesis by a paracrine or autocrine mechanism after acting through receptor complexes with a specific IL-6-binding protein and signal-transducing subunit (gp 130); the complexes had an inhibitory effect on antitumor immune responses (24).

An association between serum IL-6 level and disease status has been reported for colorectal carcinoma, with the serum IL-6 level increasing in a stage-related manner that correlated with survival (19-25). The incidence of large tumor size and lymph node and liver metastasis was significantly higher in patients with elevated serum IL-6 levels (24). In addition,

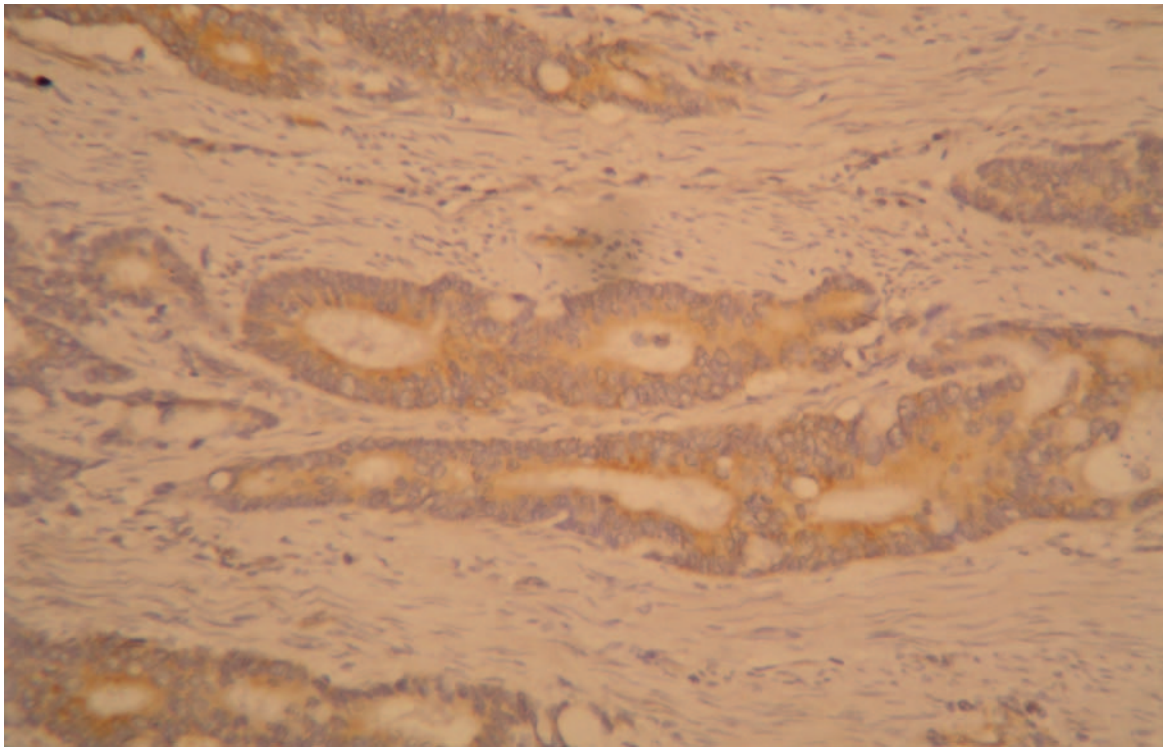

Figure 1. *Immunohistochemical staining for interleukin-6 in colorectal carcinoma. Positive immunoreactivity of interleukin-6 in the cytoplasms of carcinoma cells. Original magnification 200X.*

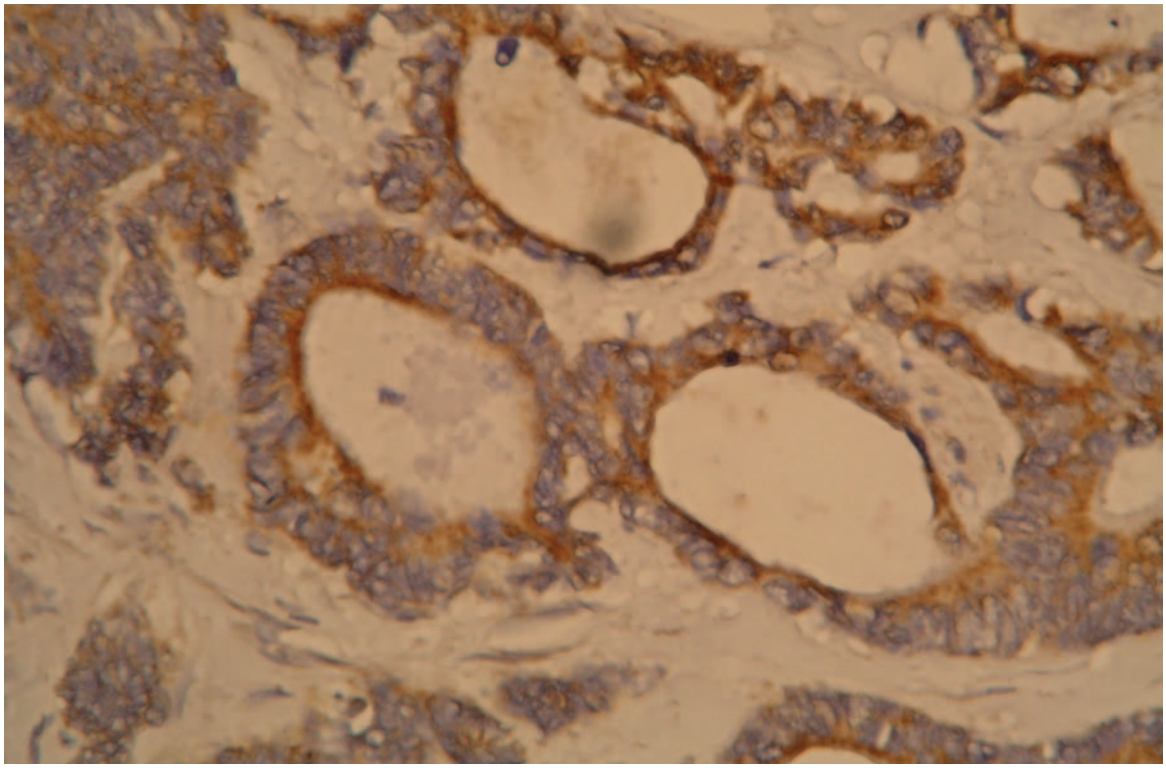

Figure 2. *Immunohistochemical staining for interleukin-6 receptor in colorectal carcinoma. Positive immunoreactivity of interleukin-6 receptor was detected not only in cytoplasm but also in cell membrane. Original magnification 400X.*

Table I. *Clinicopathological characteristics of colorectal carcinoma associated with positive and negative tissue expression of IL-6.*

	IL-6(–) (n=86)	IL-6(+) (n=74)	*P* value
Age(years)	66.6±1.5[a]	68.3±1.7[a]	0.46[b]
Gender ratio (M:F)	59 : 27	46 : 28	0.409[c]
Location of tumor			0.268[d]
Cecum/ ascending	24	19	
Transverse	2	2	
Descending	3	1	
Sigmoid	25	13	
Rectum	32	39	
Differentiation			0.196[d]
Well	17	23	
Moderate	56	42	
Poor	8	8	
Mucinous	5	1	
CEA			0.005[c]
≤5 ng/ml	61	37	
>5 ng/ml	23	36	
Mucin production			0.265[c]
Positive	15	8	
Negative	71	16	
Maximum size of tumor	5.1±0.25	5.4±0.29	0.338[b]
Growth characteristics			1.000[c]
Ulcerative	68	59	
Protruding	18	15	
Lymph node metastasis			0.049[c]
Positive	38	45	
Negative	48	29	
TNM stage			0.035[d]
I	1	0	
II	44	26	
III	35	32	
IV	6	15	
Lymphatic invasion			1.000[c]
Positive	26	22	
Negative	60	52	
Vascular invasion			0.049[c]
Positive	17	25	
Negative	69	49	
Perineural invasion			0.338[c]
Positive	23	25	
Negative	63	49	
IL-6 serum level			0.122[c]
≤12 pg/ml	23	20	
>12 pg/ml	14	29	

CEA, carcinoembryonic antigen; IL-6, interleukin-6.
[a]mean±standard deviation.
[b]Two sample *t*-test.
[c]Fisher's exact test.
[d]Chi-square test.

Table II. *Clinicopathological characteristics of colorectal carcinoma associated with positive and negative tissue expression of IL-6R.*

	IL-6R(–) (n=103)	IL-6R(+) (n=56)	*P* value
Age(years)	66.7±1.3[a]	68.5±2.1[a]	0.443[b]
Gender ratio (M:F)	68 : 35	36 : 20	0.862[c]
Location of tumor			0.056[d]
Cecum/ ascending	35	7	
Transverse	3	1	
Descending	3	1	
Sigmoid	24	14	
Rectum	38	33	
Differentiation			0.572[d]
Well	26	14	
Moderate	60	37	
Poor	12	4	
Mucinous	5	1	
CEA			0.229[c]
≤5 ng/ml	66	31	
>5 ng/ml	34	25	
Mucin production			1.000[c]
Positive	15	8	
Negative	88	48	
Maximum size of tumor	5.4±0.24	4.9±0.3	0.146[b]
Growth characteristics			0.564[c]
Ulcerative	80	46	
Protruding	23	10	
Lymph node metastasis			0.868[c]
Positive	53	30	
Negative	50	26	
TNM stage			0.837[d]
I	1	0	
II	46	23	
III	43	25	
IV	13	8	
Lymphatic invasion			0.716[c]
Positive	32	15	
Negative	71	41	
Vascular invasion			1.000[c]
Positive	27	14	
Negative	76	42	
Perineural invasion			0.473[c]
Positive	29	19	
Negative	74	37	

CEA, carcinoembryonic antigen; IL-6R, interleukin-6 receptor.
[a]mean±standard deviation.
[b]Two sample *t*-test.
[c]Fisher's exact test.
[d]Chi-square test.

tumor concentrations of IL-6 have been reported to correlate with serum IL-6 concentration in peripheral venous blood (21). These findings support the hypothesis that the serum IL-6 level reflects the IL-6 content in the tumor and that increased circulating serum level may predict survival prospects. However, one previous study reported that serum IL-6 level was not an independent prognostic factor (24). Identification of a definite role for serum IL-6 is difficult due to multiple sources of the cytokine.

This study examined the expression of IL-6 in tumor cells and revealed a significant correlation with tumor stage. It was found that IL-6 was expressed in the cytoplasm of tumor cells.

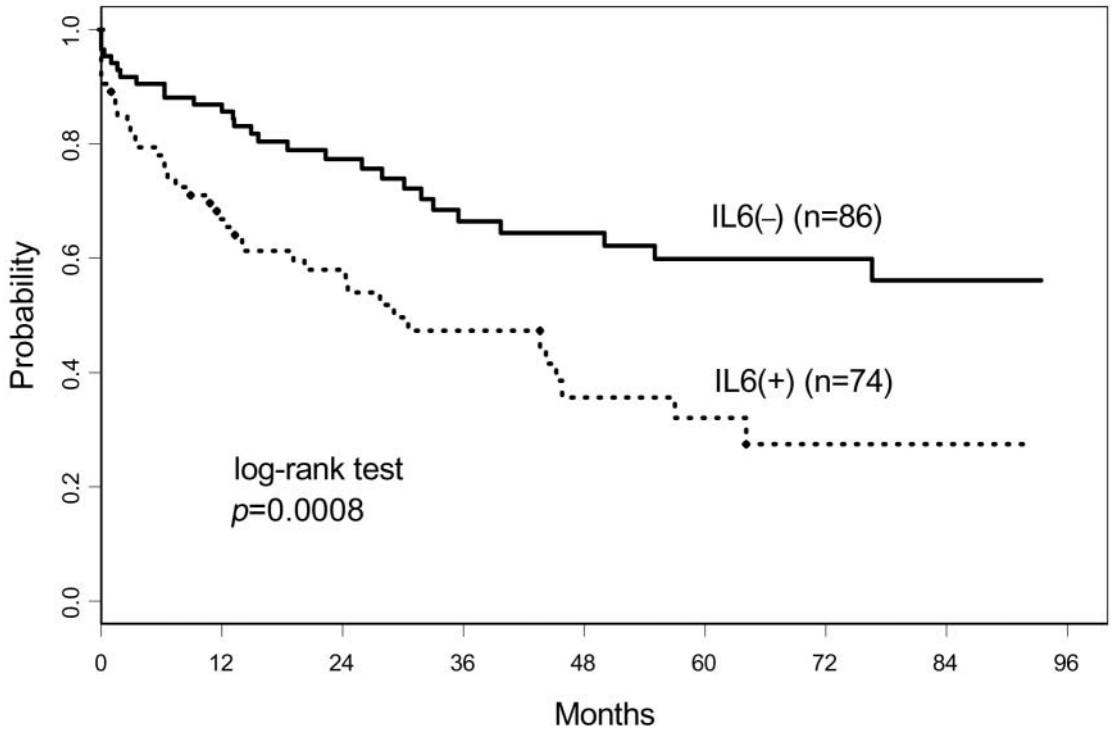

Figure 3. *Survival curve of patients who underwent colorectal resection. The survival rate in the group without IL-6 tissue expression was more favorable and the group with IL-6 tissue expression had lower survival.*

Expression of IL-6R was located not only in cytoplasm, but in cell membranes. Kinoshita *et al*. (21) reported that 60% and 65% of colorectal carcinoma samples had positive expressions of IL-6 and IL-6R. In this series, only 46.3% and 35% of specimens were positive for IL-6 and IL-6R expression, respectively. The discrepancy between reports may be due to differences in the usage of polyclonal antibodies in immunohistochemistry. Moreover, we found that more patients with advanced-stage disease had a significantly high tumor expression of IL-6. Additionally, vascular invasion correlated with tumor overexpression of IL-6. Overexpression of IL-6 in tumor also correlated with poor survival. These findings are consistent with previous observations that high serum IL-6 levels were associated with poor prognosis (19-25). However, the current study is the first to report that the tissue expression of IL-6 also directly predicts poor prognosis in colorectal carcinoma. The intention was also to clarify the role of IL-6R in this patient series. Unfortunately, IL-6R did not show any association with clinicopathological factors.

Consistent with a previous study (21), tumors in our series with IL-6 immunoreactivity had a higher incidence of IL-6R immunoreactivity. These findings suggest an interaction between IL-6 and IL-6R in tumors. In the current study, the tissue expression of IL-6 did not correlate with the serum IL-6 level. These results contradict those of Kinoshita *et al*.,

Table III. *Relationship between IL-6 and IL-6R expression in colorectal carcinoma tissue.*

	IL-6R		Total
	–	+	
IL-6			
–	66	19	85
+	37	37	74
Total	103	56	159

$p<0.001$, Fisher's exact test.

Table IV. *Results of univariate and multivariate analysis of factors potentially associated with survival in patients with colorectal carcinoma.*

	P-value		Hazard ratio	95% CI
	Univariate	Multivariate		
CEA	<0.0001	0.040	1.76	1.025 ~ 3.02
TNM stage	<0.0001	<0.0001	3.351	2.161 ~ 5.20
Perineural invasion	0.0003	0.170	1.878	1.11 ~ 3.18
Tumor IL-6	0.0008	0.0097	1.963	1.177 ~ 3.27

CI: confidence interval.

who found significant correlation between serum and tissue IL-6 concentrations. However, this difference might be explained by a different analytic technique (21). Kinoshita *et al*. evaluated homogenized specimens with enzyme-linked immunosorbent assay (ELISA). Observed IL-6 levels were produced not only by tumor cells, but also by stromal cells and macrophages in their study.

In the present study, survival was significantly lower in patients with IL-6 overexpression than in patients without IL-6 expression. Multivariate analysis with logistic regression revealed that elevated preoperative CEA level, advanced TNM stage, and tumor IL-6 overexpression were significant independent predictors of poor prognosis. These findings suggest that tumor IL-6 overexpression is an important prognostic factor for colorectal cancer. These results agree with findings of previous studies with gastric and prostate cancer (28, 29). This study also found IL-6 that expression in tumor was associated with advanced stage. Huang *et al*. (28) reported, for gastric cancer, that IL-6 overexpression in tumor was associated with high vascular endothelial growth factor and angiogenesis. These studies provide evidence that IL-6 of tumor origin may participate in modulating the progression of malignancies.

This study focused on the tumor expression of IL-6 in colorectal carcinoma. The clinical importance of IL-6 in the tumor exceeds that of the serum level as a predictor of prognosis (24, 25). Serum IL-6 level is affected by physiological and immunological reactions and tumor-stroma reactions. It is difficult to evaluate the clinical significance of cytokines accurately. Increased expression of IL-6 was found to correlate with worse prognosis and could be regarded as an independent prognostic factor. However, to elucidate the relationship between IL-6 in colorectal carcinoma and alteration in signal molecules or oncogenes involved with metastasis, will require further investigation.

In conclusion, the current study disclosed that the tissue expression of IL-6 in colorectal carcinoma was correlated with disease progression, elevated preoperative CEA level, and vascular invasion by the tumor, which suggests that IL-6 in a tumor may be a useful prognostic marker in the clinical management of patients.

Acknowledgements

The authors thank Miss Ya-Yu Chang and Dr. Yung-Chi Hou of Hsin-Chu Hospital, Taiwan, for their excellent technical and diagnostic help in immunohistochemistry.

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Received April 26, 2006
Accepted June 20, 2006