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**IMMUNOHISTOCHEMICAL DEMONSTRATION OF THYMIDYLATE
SYNTHASE (TS), p53 PROTEIN AND BCL-X_L PROTEIN IN COLORECTAL
CANCER WITH PREOPERATIVE PERORAL CHEMOTHERAPY: TS AS
MARKER OF UNRESPONSIVENESS TO 5-FLUOROURACIL**

*Shingo Kamoshida,*¹ Hiroshi Matsuoka,² Atsuji Matsuyama,¹
Ryoichi Shimomura,¹ Morito Maruta,² and Yutaka Tsutsumi¹*

Departments of ¹Pathology and ²Surgery, Fujita Health University School of Medicine,
Toyoake, Aichi 470-1192, Japan

Key Words: Bcl-X_L protein, cancer, chemoresistance, colorectal malignancies, 5-fluorouracil, grading of colorectal tumors, immunoperoxidase staining, inhibition of apoptosis, p53 protein, selection of patients, thymidylate synthase (TS).

Abstract

High expression of thymidylate synthase (TS), p53 protein and bcl-X_L protein has been suggested to be associated with chemoresistance of colorectal malignancies. The significance of TS, p53 protein and bcl-X_L protein as predictive parameters of effects of 5-fluorouracil (5-FU)-based chemotherapy on colorectal cancers was evaluated. Immunoperoxidase staining of TS, p53 protein and bcl-X_L protein was performed on formalin-fixed, paraffin-embedded, biopsied and resected specimens from 37 patients with advanced colorectal cancers, preoperatively treated with 5-FU derivatives *per os*. Suitable antigen retrieval was applied to the respective markers. Histologically, six tumors responded to the chemotherapy, while the remaining 31 tumors did not. Thirty-

Author for correspondence. * TEL: +81-562-93-2428; FAX: +81-562-93-3063; E-mail: skamo@fujita-hu.ac.jp. Manuscript received 07/22/03; accepted 08/18/03.

one control colorectal cancer cases without preoperative treatment were also analyzed. When more than one third of the cancer cells were stained, the lesions were considered high for antigen expression. High TS expression in the resected tumors was seen in 23 (74%) of 31 histologic non-responders but none in six responders. TS expression in preoperative biopsies and resected specimens was concordant in 80% of cases when two or more biopsy specimens were available. The rate of high TS expression was comparable in the control non-treated group. There was no difference between chemotherapeutic effects and the expression of p53 protein or bcl-X_L protein. Chemoresistance to 5-FU and 5-FU derivatives can be predicted by immunohistochemical detection of high TS expression in biopsy samples. This provides us with a useful guide for preoperative selection of patients unresponsive to 5-FU-based chemotherapy. Expression of p53 protein and bcl-X_L protein is unsatisfactory for predicting the 5-FU resistance.

Introduction

Colorectal cancer is one of the most common cancers worldwide, and a considerable number of patients are subject to chemotherapy, when the diagnosis was made in an advanced stage. 5-Fluorouracil (5-FU) has widely been used in the treatment of solid tumors including colorectal cancer (Bleiberg 1996). However, the response rate of colorectal cancer to this agent does not exceed 30%, and the effect of postoperative adjuvant chemotherapy remains unsettled. The preoperative identification of patients unresponsive to 5-FU leads to the avoidance of unpleasant side effects, such as skin pigmentation, diarrhea and bone marrow suppression, and thus other regimens, alternatively, should be applied to these patients. However, there have been no clinical parameters predicting the responsiveness or unresponsiveness to the 5-FU-based chemotherapy.

Thymidylate synthase (TS) catalyzes methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), an essential step for DNA synthesis (Pinedo and Peters 1988). Anti-cancer effects of 5-FU are biochemically mediated through its metabolic conversion by two additional enzymes (thymidine phosphorylase and thymidine kinase) into fluorodeoxyuridine monophosphate (FdUMP), which eventually forms a ternary covalent complex with TS and reduced folic acid, to result in

the inhibition of TS activity. Figure 1 summarizes the catabolic pathways of 5-FU and 5-FU derivatives

Figure 1. The catabolic pathways of 5-fluorouracil and its derivatives in relation to the damage on the DNA synthesis. DPD, dihydropyrimidine dehydrogenase; 5-FU, 5-fluorouracil; UFT, tegafur and uracil; 5'-DFUR, 5'-deoxyfluorouridine; TP, thymidine phosphorylase; OPRT, orotic acid phosphoribosyl transferase; FudR, fluorodeoxyuridine; FUMP, 5-fluorouridine monophosphate; TK, thymidine kinase; FdUMP, fluorodeoxyuridine monophosphate; TS, thymidylate synthase; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate

It has been suggested that high expression of TS is associated with the 5-FU resistance of colorectal cancer cells and a poor prognosis of patients (Cascinu, Aschele *et al.*, 1999, Cascinu, Catalano *et al.*, 2000, Corsi, Ciaparrone *et al.*, 2002, Johnston, Fisher *et al.*, 1994, Johnston, Lenz *et al.*, 1995, Leichman, Lenz *et al.*, 1997, Lenz, Danenberg *et al.*, 1998, Lenz, Hayashi *et al.*, 1998, Paradiso, Simone *et al.*, 2000, Tomiak, Vincent *et al.*, 2001, Yamachika, Nakanishi *et al.*, 1998). Immunohistochemical analysis of TS expression has been done in a number of reports, but all in retrospective analyses (Cascinu, Aschele *et al.*, 1999, Cascinu, Catalano *et al.*, 2000, Corsi, Ciaparrone *et al.*, 2002, Johnston, Fisher *et al.*, 1994, Johnston, Lenz *et al.*, 1995, Lenz, Danenberg *et al.*, 1998, Paradiso, Simone *et al.*, 2000, Tomiak, Vincent *et al.*, 2001, Yamachika, Nakanishi *et al.*, 1998).

Inhibition of apoptosis is one of the mechanisms responsible for the chemoresistance of cancer cells (Dive and Hickman 1991). p53 protein and bcl-2 family proteins are critical regulators of apoptosis (Harris 1996, Korsmeyer 1999). Tumor suppressor p53 protein is a pleiotropic molecule with plurifunctions. p53 protein normally functions as either an initiator of repair of the damaged sense DNA or a trigger of the apoptotic pathway if a sufficient level of damage takes place within the cell (Harwood, Frazier *et al.*, 1996, Leonard, Canman *et al.*, 1995). Inactivation or mutation of the p53 gene is allegedly associated with the enhancement of cellular resistance to DNA-damaging agents (Benhattar, Cerottini *et al.*, 1996, Bunz, Hwang *et al.*, 1999, Lowe, Schmitt *et al.*, 1993, Zheng, Wang *et al.*, 1999). It has been suggested that the p53 gene assay can be employed to predict the responsiveness of cancer to chemotherapy. Immunohistochemical overexpression of p53 protein is frequently observed in colorectal cancers, but its clinical significance as a predictive factor is still conflicting (Brett, Pickard *et al.*, 1996, Cascinu, Catalano *et al.*, 2000, Lenz, Danenberg *et al.*, 1998, Paradiso, Rabinovich *et al.*, 1996, Paradiso, Simone *et al.*, 2000).

Among members of the bcl-2 family, bcl-2 and bcl-X_L suppress apoptosis, leading to the increased resistance to anticancer agents. Reportedly, bcl-X_L protein is more crucial as an apoptosis-inhibiting factor than bcl-2 protein in colorectal cancer tissues (Maurer, Friess *et al.*, 1998), and thus, is an important factor for the 5-FU resistance in colonic cancer cell lines (Nita, Nagawa *et al.*, 1998, Nita, Ono-Nita *et al.*, 2000, Violette, Poulain *et al.*, 2002).

Simple and inexpensive immunohistochemical approaches make it possible to detect specific antigens in routinely available, paraffin-embedded tissues in any laboratory worldwide. Antigen retrieval techniques play an important role therein. In general, the optimal conditions for the antigen retrieval, such as the combination of retrieval solution and the method of heating, have been determined in respective laboratories (Miller, Swanson *et al.*, 2000). Recently, we reported (Kamoshida, Matsuoka *et al.*, 2003) that the most suitable and reproducible method for retrieving the antigenicity of TS was the pressure cooking in 1 mM ethylenediaminetetraacetic acid (EDTA) solution, pH 8.0.

In order to identify patients unresponsive to 5-FU-based chemotherapy, we immunohistochemically investigated the relationship between chemotherapeutic effects of 5-FU and the expression of TS, p53 protein and bcl-X_L protein in biopsied and resected specimens of advanced colorectal cancers, preoperatively treated with 5-FU derivatives per os. The antigens were demonstrated in formalin-fixed, paraffin-embedded sections after appropriate antigen retrieval sequences. We here present the usefulness of the immunohistochemical assessment of TS for predicting the chemoresistance to 5-FU and 5-FU derivatives.

Materials and Methods

Patients and specimens

The population studied consisted of 37 patients (23 men and 14 women; mean age 60.3 years with a range from 34 to 81 years; median 60). All the patients had undergone surgical resection for advanced colorectal cancer in 2001 at the Department of Surgery, Fujita Health University Hospital, Toyoake, Japan. The clinicopathologic features are summarized in Table I. All the patients received per oral administration of 5-FU derivatives, tegafur and uracil (UFT, 600 mg/m² per day for 2 weeks) or 5'-deoxyfluorouridine (5'-DFUR, 800 mg/m² per day for 2 weeks) until one day before operation. Informed consent for the administration of preoperative chemotherapy and also for the use of tumor tissue for analyzing antigen expression was obtained from all the patients.

Preoperative biopsied specimens and surgically resected materials were routinely fixed in 10% formalin and embedded in paraffin wax. Sections, 3µm-thick, were cut and mounted on aminopropyltriethoxysilane slides, and stained with hematoxylin and eosin (HE), in order to assess histopathologic features and the responsiveness to the preoperative chemotherapy under a light microscope.

Preoperative biopsy specimens and surgically resected materials from control patients without giving preoperative chemotherapy (n=31) were similarly analyzed.

Histologic assessment of chemotherapeutic effects

Chemotherapeutic effects were histologically evaluated on the basis of the Japanese Classification of Colorectal Carcinoma (Japanese Society for Cancer of the Colon and Rectum 1997). Major grading (grades 0-3) and additional minor grading for grade 1 (grades 1a and 1b) were employed, based upon the degree of necrosis or disappearance of tumor cells in the lesion; grade 0: no change, grade 1: mild change (grade 1a: necrosis or disappearance of the tumor seen in less than 1/3 of the entire lesion, and grade 1b: necrosis or disappearance of the tumor seen in more than 1/3 but less than 2/3 of the entire lesion), grade 2: moderate change (necrosis or disappearance of the tumor seen in more than 2/3 of the entire lesion, but still with remaining viable tumor cells), and grade 3: severe change (no viable tumor cells remain). Patients with grades 1b and 2 were categorized as the histologic responders, and the patients with grades 0 and 1a as the histologic non-responders, on the basis of the contents of a previous report (Fujii 1999). No grade 3 lesion was observed.

Immunohistochemistry

In addition to paraffin blocks of biopsy specimens, one or two representative blocks of the resected tumor principally containing tumor-nontumor junction were chosen for immunohistochemical analysis. Consecutive sections were evaluated for the expression of TS, p53 and bcl-X_L, employing the universal immuno-enzyme polymer method. The sections were deparaffinized with xylene, and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with 0.03% hydrogen peroxide in methanol for 30 min at room temperature. Antigen retrieval was applied using a pressure cooker (Delicio 6L, T-FAL, France) for 10 min. Optimal soaking solutions were selected for the respective markers determined by preliminary experiments; 1 mM EDTA solution, pH 8.0 for TS (Kamoshida, Matsuoka *et al.*, 2003), and 10 mM citrate buffer, pH 7.0 for p53 protein and bcl-X_L protein. After pressure cooking, the sections were left for cooling in the soaking solution for 30 min on the table. The sections were then incubated with the primary antibody against TS (rabbit polyclonal, 1:200 x dilution; Taiho Pharmaceutical, Saitama, Japan), p53 protein (mouse monoclonal, clone DO-7, 1:400 dilution; Dako

Cytomation, Glostrup, Denmark) or bcl-X_L protein (mouse monoclonal, clone 2H12, prediluted; Zymed Laboratories, CA, USA) overnight at room temperature. After rinsing in 10 mM phosphate-buffered saline (PBS), pH 7.2, the sections were incubated with the universal immunoperoxidase polymer, anti-mouse and -rabbit (Histofine[®] Simple Stain MAX PO; Nichirei, Tokyo, Japan) for 30 min at room temperature. The reaction products were visualized in 50 mM Tris-HCl buffer, pH 7.6, containing 50 mg/dl diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide. The nuclei were lightly counterstained with Mayer's hematoxylin. No immunoreactions were seen in negative control slides, in which the primary antibody was replaced with PBS.

For evaluating TS and bcl-X_L immunoreactivity, tumors were classified into two groups: *low expression* (negative or positive but less than 1/3 of tumor cells) versus *high expression* (positive more than 1/3 of tumor cells). For p53 protein, low and high expressions were replaced with negative and positive, respectively. The HE-stained and immunostained sections were independently reviewed by three investigators (SK, AM and RS), without prior knowledge of clinical data of the patients. When the discrepancies existed, discussion on a multi-headed microscope was made to adjust the differences.

Statistical analyses

Fisher's exact probability test was employed for determining the statistical significance of correlations between histologic chemotherapeutic effects and the antigen expression, the gender of the patients, tumor location, histologic grade, distant metastasis or the kind of administered agent. Correlation between the chemotherapeutic effects and the depth of invasion, lymph node metastasis, clinical stage or Dukes' classification was analyzed using the chi-square test. The Student's t-test was employed for evaluating the association of the chemotherapeutic effects with the age of the patients. Categories consisting of less than 5% (i.e. two cases or less) of the total number were combined into appropriate categories (Stages I and II), or excluded from the analysis ("mucinous carcinoma" in histology). P-values <0.05 were considered statistically significant.

Figure 2. Endoscopic findings before preoperative peroral 5-FU administration (a) and macroscopic findings of the resected tumor after the preoperative chemotherapy (b). Chemotherapeutic effect is grossly evident. The histologic grade for chemotherapeutic response in this case was 2.

Figure 3. Histologic features of resected colon cancer responsive to 5-FU derivative (UFT). (a): HE staining, (b): TS immunoreactivity. Chemotherapy-induced histologic changes include disintegration of glandular structures with marked inflammatory cell infiltration and degeneration of the tumor cells. Only a few cancer cells (arrowhead) and plasma cells (arrows) in the stroma are immunoreactive for TS.

TABLE I
Relationship between chemotherapeutic effects and clinicopathologic features of patients

Clinicopathologic features	Histologic Responders (n=6)	Histologic non-responders (n=31)	p-value	Control patients (n=31)
Age (yrs.)				
Median	71.5	60.0	0.17	63.0
Mean	66.5	59.1		63.4
Gender				
Male	2	21	0.17	16
Female	4	10		15
Tumor location				
Colon	3	17	>0.99	14
Rectum	3	14		17
Histologic grade ^{*,†}				
G1	1	18	0.08	18
G2	5	12		11
Depth of invasion*				
T2	2	6	0.26	3
T3	0	10		11
T4	4	15		17
Lymph node metastasis*				
N0	4	13	0.42	16
N1	1	14		7
N2	1	4		8
Distant metastasis*				
M0	5	27	>0.99	26
M1	1	4		5
Clinical stage*				
I and II	4	11	0.30	13
III	1	15		13
IV	1	5		5
Dukes' classification				
A	1	3	0.40	2
B	3	8		11
C	1	17		16
D	1	3		2
Agent administered				
UFT	4	20	>0.99	-
5'-DFUR	2	11		-

*According to the TNM Classification of Malignant Tumors (Reference, Sobin and Wittekind 1997); [†]Histologic grade was evaluated in 36 of 37 tumors treated with preoperative chemotherapy. Two cases of G3 were also included in the control patient group; UFT, tegafur and uracil; 5'-DFUR, 5'-deoxyfluorouridine.

Results

Chemotherapeutic effects and clinicopathologic features

Two cases with the histologic grade 2 effects showed considerable tumor shrinkage under a colonofiberscope, as shown in Figure 2. Chemotherapeutic responders were histologically selected in a total of six (16%) out of 37 cases (grade 1b: four cases and grade 2: two cases) (Figure 3a).

Thirty-one cases (84%) were categorized in non-responders (grade 0: 22 cases and grade 1a: nine cases) (Figure 4a). Any of the clinicopathologic parameters showed no difference between the responders and non-responders (Table I).

The control group without preoperative chemotherapy (n=31) also showed almost comparable clinicopathologic features, as shown in Table I.

Chemotherapeutic effects and expression of TS, p53 and bcl-X_L in resected colorectal tumors preoperatively treated with 5-FU derivatives

Table II shows the relationship between the preoperative chemotherapeutic effects and the expression of TS, p53 protein or bcl-X_L protein in the resected tumors preoperatively treated with 5-FU *per os*. High TS levels were detected in none of the responders (Figure 3b) and 22 lesions (71%) of the non-responders (Figure 4b) ($p = 0.0013$). All responders showed low TS expression, whereas nine (29%) of 31 non-responders were also categorized in the low TS group. TS immunoreactivity was observed in the cytoplasm of the cancer cells with a heterogeneous distribution of positivity. Commonly, a variable number of positive cells were intermingled with negative or equivocally positive cells. Crypt epithelial cells located in the basal half of the mucosa, as well as such non-epithelial cells as germinal center lymphocytes, plasma cells, endothelial cells, fibroblasts and smooth muscle cells, were also immunoreactive. There were little differences in the staining pattern between invasive and non-invasive components within the same tumors.

Expression of p53 protein was observed in the nuclei of the cancer cells in four lesions (67%) of the responders and in 20 lesions (65%) of the non-responders (Figure 5a). Bcl-X_L protein was highly expressed in three lesions (50%) of the responders and in 14 lesions (45%) of the non-responders. The bcl-X_L antigen was distributed in the

cytoplasm of cancer cells, and frequently showed a stronger staining pattern in the invasive component of the tumors (Figure 5b). Non-neoplastic mucosa was negatively or equivocally immunoreactive for p53 protein and bcl-X_L protein, and macrophages and plasma cells in the tumor tissue were occasionally positive for bcl-X_L protein. There was no significant difference in the frequency of expression of p53 protein or bcl-X_L protein between the responders and non-responders.

TABLE II

Relationship between chemotherapeutic effects and expression of TS, p53 and bcl-X_L in surgically resected tumors

Antigen expression	Histologic responders (%)	Histologic non-responders (%)	<i>p</i> -value
TS			
Low	6 (100)	8 (26)	0.0013
High	0 (0)	23 (74)	
p53 protein			
Negative	2 (33)	11 (35)	>0.99
Positive	4 (67)	20 (65)	
Bcl-X _L protein			
Low	3 (50)	17 (55)	>0.99
High	3 (50)	14 (45)	

TABLE III

Relationship between expression of p53 or bcl-X_L and chemotherapeutic effects in 14 resected tumors with low TS expression

Expression of p53 and bcl-X _L	Histologic responders (%)	Histologic non-responders (%)	<i>p</i> -value
p53 protein			
Negative	2 (33)	2 (25)	>0.99
Positive	4 (67)	6 (75)	
Bcl-X _L protein			
Low	3 (50)	3 (38)	>0.99
High	3 (50)	5 (63)	

Table III summarizes the analysis of cases with low TS expression. p53 protein positivity was observed in four tumors (67%) in the responders and six (75%) in the non-responders. High bcl-X_L expression was noted in three (50%) tumors in the responders

and five (53%) of the non-responders. Expression of p53 protein and bcl-X_L protein was hence not associated with the chemoresistance even in the tumors with low TS expression.

There were no significant differences in the expression of TS, p53 protein and bcl-X_L protein between the preoperatively treated tumors and control untreated tumors (Table IV).

TABLE IV

Comparative expression of TS, p53 and bcl-X_L in tumors resected from preoperatively treated patients and from control untreated patients

Antigen expression	Preoperatively treated cases (%)	Control untreated cases (%)	<i>p</i> -value
TS			
Low	14 (38)	12 (39)	>0.99
High	23 (62)	19 (61)	
p53 protein			
Negative	13 (35)	8 (26)	0.44
Positive	24 (65)	23 (74)	
Bcl-X _L protein			
Low	20 (54)	15 (48)	0.81
High	17 (46)	16 (52)

Figure 4. Histologic features of resected colon cancer unresponsive to 5-FU derivative (UFT). (a): HE staining, (b): TS immunoreactivity. Little histologic changes are discernible. TS is highly expressed in a majority of cancer cells, in addition to plasma cells in the stroma (arrows).

Comparative TS expression in preoperative biopsy specimens and resected materials

Adequate biopsy material was available in 30 of the 37 cases with preoperative chemotherapy and in 25 of the 31 control untreated cases. We compared the expression of TS in the biopsy specimens with the positivity in the resected tumors. High TS expression was seen in 17 (57%) of 30 biopsy specimens from the non-responders but none from the responders. Seventeen (68%) of 25 biopsy specimens from the control untreated patients were also showed high TS expression. Table V demonstrates a concordant pattern between the preoperative biopsy specimens and the resected materials. Two (40%) of five preoperatively treated cases and four (57%) of seven control untreated cases were concordant, when the number of biopsied tumor fragment was only one. Twenty (80%) of 25 treated cases and 15 (83%) of 18 control cases were concordant, when two or more biopsy samples were obtained and analyzed. Although no statistical significance was shown ($p = 0.10$ in the treated cases, and $p = 0.30$ in the control cases), it was suggested that the larger the number of biopsy specimens is, the more justified can we be in the involvement of TS expression in tumor resistance.

Figure 5. Immunoreactive p53 protein (a) and bcl-X_L protein (b) in resected colon cancer responsive to 5-FU derivative. Overexpression of these proteins is similarly observed in tumors of both the responders and non-responders. Bcl-X_L protein shows increased expression in the invasive tumor component (arrow).

Correlation of TS expression between cancer tissues and the adjacent non-neoplastic mucosa

Table VI exhibits the correlation of TS expression between the cancer tissue and the adjacent non-neoplastic mucosa. TS immunoreactivity was positive in the non-neoplastic crypt epithelium, particularly in the basal half of the mucosa in 30 (83%) of 36 preoperatively treated cases and in 21 (75%) of 28 control untreated cases. No significant difference was detected ($p = 0.53$).

TABLE V

Correlation of TS expression between biopsied and resected tumors, according to the number of biopsy fragments

Biopsy		TS expression in resected tumor		Concordance (%)
No. of fragments	TS expression	Low	High	
Preoperatively treated cases (n=30)				
1 (n=5)	Low	1	1	2 (40)
	High	2	1	
2 or more (n=25)	Low	8	3	20 (80)
	High	2	12	
Control untreated cases (n=25)				
1 (n=7)	Low	0	2	4 (57)
	High	1	4	
2 or more (n=18)	Low	6	0	15 (83)
	High	3	9	

TABLE VI

Correlation of TS expression between cancer tissue and the adjacent non-neoplastic crypt epithelium

TS expression in cancer tissue	TS expression in adjacent non-neoplastic crypt epithelium		Concordance (%)
	Negative or weakly positive	Positive	
Preoperatively treated cases (n=36)			
Low	1	12	19 (51%)
High	5	18	
Total	6 (17%)	30 (83%)	
Control untreated cases (n=28)			
Low	4	8	17 (61%)
High	3	13	
Total	7 (25%)	21 (75%)	

The concordance of TS expression between the non-neoplastic epithelia and cancer tissues was 51% in the treated cases and 61% in the control cases, respectively ($p = 0.61$). Strong TS expression in the non-epithelial components was consistently observed in all the specimens.

Discussion

It is well known that advanced colorectal cancers differently respond to chemotherapy. At the present time, however, there are no precise methods by which the response to anti-cancer agents can be predicted for the individualized “tailor-made” chemotherapy. The preoperative chemotherapy may provide information on the responsiveness of a given tumor to a particular drug regimen. Fujii (1999) employed a short administration period (10 days) of UFT for the preoperative treatment of colorectal cancer, and demonstrated that the histopathologic analysis of resected specimens following preoperative chemotherapy can predict chemosensitivity to postoperative 5-FU-based chemotherapy for preventing the recurrence. Matsuura, Fukuda *et al.*, (2000) described that preoperative 5-FU suppository administration induced apoptosis and suppressed angiogenesis in colorectal cancers in a p53-independent manner. To the best of our knowledge, the present study is the first attempt to predict the cancer resistance to 5-FU, by means of immunostaining for TS, p53 protein and bcl-X_L protein in colorectal cancers treated with preoperative, peroral 5-FU-based chemotherapy.

p53 protein expression was detected in 67% of the responders and 65% of the non-responders, indicating no relationship between p53 expression and histologic response to 5-FU derivatives. Several studies have demonstrated that p53 expression shows no association with the clinical response to biomodulated 5-FU chemotherapy (Cascinu, Catalano *et al.*, 2000, Paradiso, Rabinovich *et al.*, 1996, Paradiso, Simone *et al.*, 2000). However, caution should be paid when interpreting these data. Immunostaining does not necessarily distinguish the accumulation of abnormal p53 protein due to gene mutation from the overexpression of normal protein (Lenz, Hayashi *et al.*, 1998, Momand,

Zambetti *et al.*, 1992). In fact, it has been reported that mutation or disruption of the p53 gene is associated with low chemosensitivity to 5-FU (Bunz, Hwang *et al.*, 1999, Zheng, Wang *et al.*, 1999). p53-independent regulation of apoptosis in colorectal cancers has been shown in the previous *in vitro* studies (Bracey, Miller *et al.*, 1995). It is thus still debatable whether or not p53 has clinical significance in 5-FU-based chemotherapy against colorectal cancers.

Experimental studies have clarified that the regulation of apoptosis induced by anti-cancer agents involves a dynamic interplay among the bcl-2 family proteins. There are investigations demonstrating that bcl-X_L, a member of the bcl-2 family, is a strong apoptosis-inhibiting factor leading to the 5-FU resistance (Nita, Nagawa *et al.*, 1998, Nita, Ono-Nita *et al.*, 2000). The present study, however, failed to identify a positive correlation between the 5-FU chemoresistance and the high expression of bcl-X_L. Other apoptosis protectors may thus contribute to the development of the resistance to 5-FU derivatives *in vivo*. It is noteworthy that the bcl-X_L positivity tended to be stronger in the invasive components of tumors. These findings may suggest that bcl-X_L play a important role in colorectal tumor progression (Maurer, Friess *et al.*, 1998), rather than the 5-FU chemoresistance.

There are several TS assays, including the FdUMP binding assay, the dUMP assay and the measurement of TS mRNA. However, all of them require fresh or frozen tumor tissue and does not have a function of checking tissue morphology. Immunostaining of TS provides benefits, as follows. 1) This is a relatively simple and inexpensive technique and can be performed in any laboratory worldwide. 2) This detects TS protein in routinely available, paraffin-embedded tissues, retrospectively. 3) This distinguishes TS-expressing cancer cells from positively stained normal epithelial cells or stromal inflammatory cells. 4) This also allows observation of the staining pattern in relation to histologic features in various parts of tumors.

Our analysis provided further support to the concept that high TS expression is associated with the 5-FU resistance. High TS expression was observed in the lesions unresponsive to preoperative peroral 5-FU-based chemotherapy, but none in the

responders. Likewise, the majority of the previous retrospective studies indicated that patients with colorectal cancers showing high levels of TS protein (Cascinu, Aschele *et al.*, 1999, Cascinu, Catalano *et al.*, 2000, Corsi, Ciaparrone *et al.*, 2002, Johnston, Fisher *et al.*, 1994, Johnston, Lenz *et al.*, 1995, Paradiso, Simone *et al.*, 2000) and TS mRNA (Johnston, Lenz *et al.*, 1995, Leichman, Lenz *et al.*, 1997) least likely benefit from 5-FU-based postoperative adjuvant chemotherapy.

High TS expression invariably predicted the resistance to 5-FU derivatives, while low TS expression did not necessarily indicate the responsive group. All responders showed low TS expression, but certain lesions with low TS expression were unresponsive to 5-FU derivatives. Our results also indicated that the expression of p53 protein or bcl-X_L protein is not an independent predictor of the response to 5-FU derivatives and is not associated with chemotherapeutic effects even in patients with low TS expression (Cascinu, Catalano *et al.*, 2000). 5-FU-chemoresistant mechanisms in the non-responders with low TS expression require further investigations, including immunohistochemical analysis of factors influencing the effectiveness of 5-FU derivatives, such as dihydropyrimidine dehydrogenase, thymidine phosphorylase and thymidine kinase (Kralovanszky, Koves *et al.*, 2002, Nishimura, Terada *et al.*, 2002, Salonga, Danenberg *et al.*, 2000).

We demonstrated a good agreement in TS expression between preoperatively biopsied specimens and surgically resected materials, suggesting that TS immunostaining in preoperatively biopsied specimens can be utilized for the prediction of 5-FU chemoresistance. This was evident when the number of biopsy pieces was two or more. Discrepant results were probably due to the heterogeneous TS distribution seen in most cancer tissues. We also indicated that there is no difference regarding TS expression in cancer tissues and non-neoplastic mucosa between the preoperatively treated cases and control untreated cases. These results suggest that TS expression in cancer tissues and non-neoplastic mucosa may be unchanged after 5-FU-based chemotherapy; namely, no enzyme induction occurred. Several investigators have described an acute increase of TS protein levels in the time- and dose-dependent manner after the exposure of colonic

cancer cells to 5-FU (Backus, Dukers *et al.*, 2001, Chu, Koeller *et al.*, 1993). In contrast, it has been demonstrated that preoperative chemotherapy using the 5-FU derivative, UFT, induces suppression of TS activities in colorectal cancer tissues (Sakamoto, Tsukada *et al.*, 2002).

Such possibility of enzyme induction should also be the target of future analysis, in consideration of the differences in measuring methods (immunostaining vs. immunobot), experimental models (in vivo vs. in vitro), the agents administered, the concentration of 5-FU exposed to the cancer cells, and the extent of the entrapment of TS-expressing non-epithelial cells within the sample.

In conclusion, we demonstrated that high TS expression is the best predictor of the resistance to preoperative 5-FU-based chemotherapy. Our data also suggest that the immunohistochemical analysis of TS will render it possible to select patients with colorectal cancers unresponsive to 5-FU-based chemotherapy, preoperatively. The recognition of patients unresponsive to 5-FU and 5-FU derivatives allows a choice of the different postoperative treatment regimens, employing the alternative drugs acting with a different mechanism, such as irinotecan (CPT-11) and oxaliplatin (Rougier, Bugat *et al.*, 1997). In order to assess the possible role of immunostaining in predicting a benefit of adjuvant systemic chemotherapy, we are now examining the relationship between the prognosis of colorectal cancer patients and the expression of TS, p53 protein or bcl-X_L protein in the primary and recurrent tumors.

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