The expressions of p21 and pRB may be good indicators for the sensitivity of esophageal squamous cell cancers to CPT-11: Cell proliferation activity correlates with the effect of CPT-11

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Previously, we demonstrated that CPT-11 is an effective agent against esophageal squamous cell cancers (ESCC), and that the protein level of DNA topoisomerase I can be a predictor for sensitivity to CPT-11 (*Jpn J Cancer Res* 2001; 92: 1335–41). Here, we describe our search for additional predictors of sensitivity to CPT-11, mainly among cell cycle-regulating proteins, because the cytotoxicity of CPT-11 is significantly correlated with the percentage of ESCC cells in S-phase. To this end, we selected and examined the expressions of 5 proteins involved in G_1-S transition, i.e., p53, cyclin D1, p21, p27, and pRB, in 14 ESCC cell lines by western blot analysis. Among these proteins, the expression levels of p21 and pRB showed significant differences that were associated with the IC_{50} values for CPT-11 (P=0.0339 and P=0.0109, respectively). Namely, the expression of p21 or pRB independently could be a good indicator of CPT-11 efficacy in ESCC. In addition, the cell proliferation activities examined by enzyme-linked immunosorbent assay (ELISA) using 5-bromo-2′-deoxyuridine (BrdU) showed a significant correlation with the percentage of total S-phase cells (correlation coefficient=0.568, P=0.0324), and an inverse correlation with the IC_{50} values for CPT-11 (correlation coefficient= −0.601, P=0.0213). Because, as in the case of DNA topoisomerase I, the cell proliferation activity determined using BrdU shows a close relationship with the MIB-1 labeling index, immunohistochemical studies of p21, pRB, and MIB-1 in resected ESCC specimens and/or biopsy samples could make it possible to predict more precisely the sensitivity of ESCC patients to CPT-11 prior to treatment. (Cancer Sci 2004; 95: 464–468)

In recent years, many anti-cancer drugs have been developed that show potent anti-tumor activities against various experimental and clinical cancers. Moreover, combination chemotherapy using several anti-cancer drugs plays an important role in improving therapeutic efficacy. For esophageal squamous cell cancers (ESCC), *cis*-dichlorodiammineplatinum (II) (CDDP) is the key drug used for treatment, with 5-fluorouracil and/or leucovorin mainly used together as biochemical modulators.[1, 2] Recently, paclitaxel, docetaxel, and CPT-11 have been selected as therapeutic agents against ESCC, and some clinical trials using these agents together with CDDP[3–5] have indicated them to have a significant clinical impact. Anti-cancer drugs demonstrate excellent therapeutic efficacy if the cancer cells are sensitive to the drugs. However, inappropriate anti-cancer treatments could cause rapid tumor progression in addition to severe side effects. Therefore, it is very important to predict the sensitivity of a cancer to anti-cancer drugs on the basis of molecular biological characteristics to avoid ineffective or harmful treatments, as well as to improve the poor prognosis of ESCC patients.

Previously, we demonstrated that CPT-11 is an effective anti-cancer agent for ESCC, and that the protein level of DNA topoisomerase I can be used as a predictor for sensitivity to CPT-11.[6] In ESCC, several cell cycle-regulating factors, such as p53, p21, cyclin D1, and pRB, exhibit abnormal expressions, and play important roles in tumor invasion, metastatic potential, and the patients' prognosis.[7–13] Because the cytotoxicity of CPT-11 is S-phase-specific,[14] the abnormal expressions of proteins that regulate G_1-S transition through the cell cycle could have important correlations with sensitivity to CPT-11. Therefore, this study was undertaken in an attempt to find additional predictors, mainly among cell cycle-regulating proteins, for sensitivity to CPT-11. The cell proliferation activities of ESCC cell lines were also examined for a correlation with the therapeutic efficacy of CPT-11.

Materials and Methods

Cell lines. Twelve human ESCC cell lines of the TE series[15] were kindly provided by Dr. T. Nishihira of the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. EC-GI-10 was obtained from the Riken Gene Bank, and TT was from the Human Science Research Resources Bank. Ten cell lines (TT, TE-1, TE-2, TE-5, TE-8, TE-10, TE-11, TE-12, TE-13, TE-15) were established from primary ESCC lesions, and the rest were from metastatic lesions.

Cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% sterile, filtered fetal bovine serum (Moregate BioTech, Bulimba, QLD, Australia) and 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Antibiotec-Antimycotic, Gibco) at 37°C in a humidified 5% CO_2 atmosphere.

Cell cycle analysis. Cell cycle analysis was performed by fluorescence-activated cell sorting (FACS) as previously described.[16] Briefly, semi-confluent ESCC cells grown in culture dishes were suspended in 1 ml of 0.5% RNase solution at a cell concentration of 1×10^6/ml. Cells were stained with propidium iodide at a final concentration of 50 μg/ml, and analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA). The percentages of total S-phase cells were evaluated using Modfit LT ver. 3.0 for Macintosh (Verity Software House, Inc., Topsham, ME). The percentages of total S-phase cells are expressed as the means of 3 independent experiments.

Antibodies. Anti-p53 antibody (DO-1, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a dilution of 1:4000, anti-cyclin D1 antibody (DCS-6, DAKO, Glostrup, Denmark) at 1:200, anti-p21 antibody (F-5, Santa Cruz Biotechnology,

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Inc.) at 1:200, anti-p27 antibody (F-8, Santa Cruz Biotechnology, Inc.) at 1:200, and anti-pRB antibody (G3-245, Pharmingen, San Diego, CA) at 1:500. As an internal control, anti-β-actin antibody (AC-15, Sigma, St. Louis, MO) was used at 1:5000 dilution. As the secondary antibody, anti-mouse IgG-AP (Santa Cruz Biotechnology, Inc.) was used at 1:2000 dilution.

Immunoblot analysis. Protein extraction and immunoblotting were performed essentially as described previously.[6] To extract protein from cells, culture dishes containing semi-confluent cells were washed 3 times with ice-cold phosphate-buffered saline (PBS), and then the cells were harvested. Cells were lysed in buffer including 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), 5 μl/ml aprotinin, 5 μg/ml leupeptin, 5 μ*M* NaF, and 0.2 μ*M* sodium orthovanadate in NETN [20 m*M* Tris-HCl (pH 8.0), 100 m*M* NaCl, 1 m*M* EDTA, and 0.5% NP-40], and incubated on ice for 30 min. Protein extracts were obtained after centrifugation at 14,000 rpm for 15 min at 4°C, and quantified by means of the Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA).

The extracted proteins were boiled with an equal volume of 2× SDS sample buffer [2% SDS, 0.1% bromophenol blue, 100 m*M* dithiothreitol, and 10% glycerol in 50 m*M* Tris-HCl (pH 6.8)], resolved by electrophoresis in SDS-polyacrylamide gels, and transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, UK).

The membranes were blocked with 5% powdered milk in TBST [10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, and 0.075% Tween 20] for 1 h at room temperature, and then incubated overnight with a primary antibody at 4°C. The membranes were then washed 4 times with TBST, and the bound antibodies were detected using alkaline phosphatase-conjugated secondary antibodies, and developed using Immune-Star (Bio-Rad Laboratories) with a lumino-image analyzer (LAS-1000, Fuji Film, Tokyo).

The antibodies were removed by washing the membranes in removal solution [100 m*M* 2-mercaptoethanol, 2% SDS, and 62.5 m*M* Tris-HCl (pH 6.7)] at 60°C for 30 min, and the membranes were re-blotted for β-actin to confirm that equal amounts of protein were loaded.

Cell proliferation assay. The proliferation activities of the ESCC cell lines were analyzed by enzyme-linked immunosorbent assay (ELISA) using a Cell Proliferation ELISA system, version 2 (Amersham Pharmacia Biotech) according to the manufacturer's instructions. In brief, suspensions of each of the 14 ESCC cell lines at 2.5×10^5 cells/ml were seeded in 96-well microplates. After incubation at 37°C in a humidified 5% CO_2 atmosphere for 24 h, the cells were exposed to 10 μ*M* 5-bromo-2′-deoxyuridine (BrdU) solution for 2 h, followed by cell fixation, DNA denaturation and blocking. After incubation with peroxidase-labelled anti-BrdU, the wells were rinsed and 3,3′,5,5-tetramethylbenzidine (TMB) in 15% dimethylsulfoxide (DMSO) was added. The samples were mixed for 10 min, after which 1 *M* sulfuric acid was added to stop the reaction, and the absorbance was measured at 450 nm using a microplate reader (Model 550, Bio-Rad Laboratories). The cell proliferation activity values are expressed as the means of at least 3 independent experiments, each performed in 5 replicates.

Statistical evaluation. Univariate analysis was performed by means of the Mann-Whitney *U* test. The correlation test was performed using Pearson's correlation coefficient. The values of the percentage of total S-phase cells, cell proliferation activity, and IC_{50} (50% inhibition of cell growth of treated cells compared with control cells) are expressed as mean±standard deviation (SD). All statistical evaluations were performed by Stat View 5.0 for Macintosh (HULINKS, Inc., Tokyo). $P<0.05$ was considered statistically significant.

Results

Relationship between sensitivity to CPT-11 and the percentage of total S-phase ESCC cells. Because the cytotoxicity of CPT-11 is S-phase-specific,[14] we hypothesized that tumors with a high percentage of S-phase cells were more likely to be damaged by CPT-11, and that CPT-11 would be effective against such tumors. Therefore, at first, the cell cycles of 14 ESCC cell lines were analyzed by FACS (Fig. 1A). The percentages of total S-phase cells ranged from 13.213 to 45.453 with a mean±SD of 27.558±10.011. The percentages of total S-phase cells in primary tumor cell lines and metastatic tumor cell lines were 29.086±9.299 and 23.739±12.150, respectively, showing no significant difference by the Mann-Whitney *U* test.

To examine the relationship between the therapeutic efficacy of CPT-11 and the percentage of S-phase cells, the IC_{50} values for CPT-11 described previously (Fig. 2)[6] and the percentages of total S-phase cells of 14 ESCC cell lines were analyzed. An inverse correlation was observed between the IC_{50} values for CPT-11 and the percentage of total S-phase cells (correlation coefficient=−0.631, P=0.0138: Fig. 1B). This result may indicate that CPT-11 is effective against ESCC tumors with a high percentage of S-phase cells.

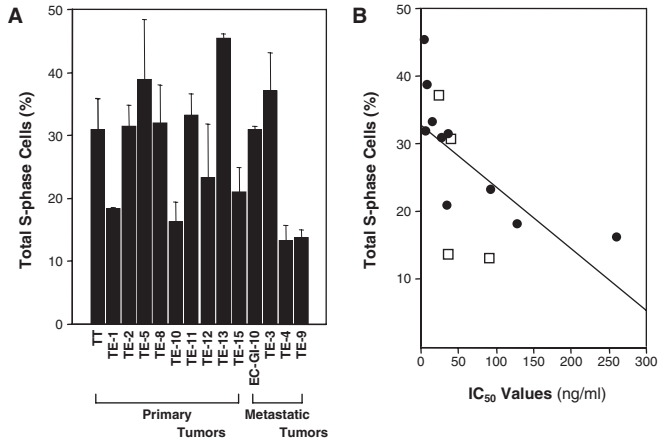

Fig. 1. Cell cycle analysis of 14 ESCC cell lines performed by FACS (A). The percentages of total S-phase cells ranged from 13.213 to 45.453 with a mean±SD of 27.558±10.011. The mean percentages of total S-phase cells in primary tumor cell lines (●) and metastatic tumor cell lines (□) were 29.086±9.299 and 23.739±12.150, respectively (B). The percentages of total S-phase cells in the 14 cell lines show an inverse correlation with the IC_{50} values for SN-38 (correlation coefficient=−0.631, P=0.0138).

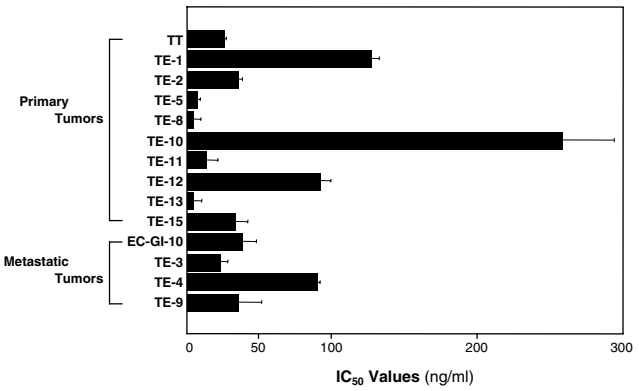

Fig. 2. IC_{50} values of 14 ESCC cell lines for SN-38, an active metabolite of CPT-11, were determined by growth inhibition assay as described before.[6]

Relationship between sensitivity to CPT-11 and the expression of cell cycle-regulating proteins. Because ESCC tumors with a high percentage of S-phase cells may respond to CPT-11, the expression levels of cell cycle-regulating proteins, especially those involved in G_1-S transition, could be good indicators for CPT-11 sensitivity. Therefore, we examined the expressions of 5 cell cycle-regulating proteins (p53, cyclin D1, p21, p27, and pRB) in 14 ESCC cell lines by western blot analysis (Fig. 3). The overexpression of p53 and cyclin D1 was detected in 8 (57.1%) and 5 (35.7%) cell lines, respectively. High levels of p21 and p27 expression were detected in 10 (71.4%) and 6 (42.9%) cell lines, respectively. There was no significant difference in the expression levels of these proteins between primary tumor cell lines and metastatic tumor cell lines (data not shown). As for pRB, 3 cell lines (TE-1, TE-10, and EC-GI-10) showed no expression, and one cell line (TE-4) showed only the hypophosphorylated form. When pRB is hypophosphorylated, the cells are either quiescent or in early G_1-phase. However, the protein samples analyzed were extracted from cycling cells in culture dishes. Therefore, it is unlikely that all TE-4 cells were in G_0 or early G_1 phase. It has been reported that some mutated forms of pRB cannot be phosphorylated, and, as a result, show functional loss.[17–20] The TE-4 protein band detected was considered to represent an aberrant and non-functional pRB. As a result, these 4 cell lines were categorized as abnormal for pRB expression. There was also no significant difference in terms of pRB expression between primary tumor cell lines and metastatic tumor cell lines (data not shown).

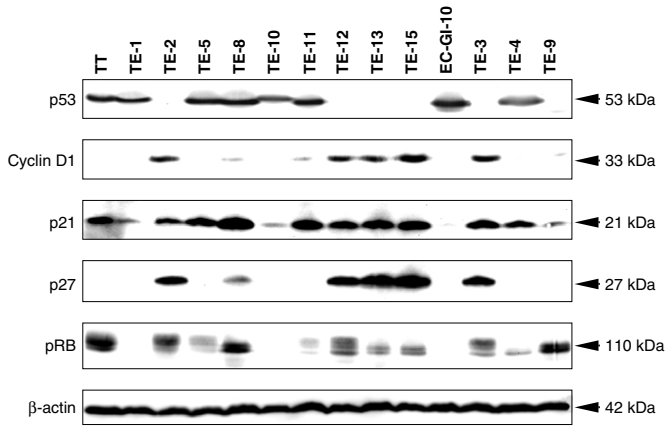

Fig. 3. Western blotting for p53, cyclin D1, p21, p27, pRB, and β-actin in 14 ESCC cell lines. Protein samples extracted from each of 14 ESCC cell lines were separated in SDS-polyacrylamide gels, and transferred to PVDF membranes. The protein bands were detected by chemiluminescence. p53 was detected as a 53 kDa band, cyclin D as a 33 kDa band, p21 as a 21 kDa band, p27 as a 27 kDa band, pRB as a 110 kDa band, and β-actin as a 42 kDa band. The overexpression of p53 and cyclin D1 was detected in 8 (57.1%) and 5 (35.7%) cell lines, respectively. High expression levels of p21 and p27 were detected in 10 (71.4%) and 6 (42.9%) cell lines, respectively. The TE-1, TE-10, and EC-GI-10 cell lines showed no pRB expression, while pRB in TE-4 cells was in a hypophosphorylated state.

Among 5 cell cycle-regulating factors, the expression levels of p21 and pRB showed significant differences in terms of the percentages of total S-phase cells by the Mann-Whitney *U* test (P=0.0477 and P=0.0339, respectively: Table 1). The percentages of total S-phase cells in the high and low p21 expression groups were 30.670±9.367 and 19.778±7.623, respectively, and the percentages of total S-phase cells showing normal and abnormal pRB expression were 30.719±9.266 and 19.656±7.756, respectively. These results suggest that ESCC cell lines showing a low expression of p21 and/or aberrant expression of pRB have significantly low percentages of total S-phase cells.

The IC_{50} values for CPT-11 in 14 ESCC cell lines have been described before.[6] Using those results, the relationship between the expression levels of cell cycle-regulating proteins and the efficacy of CPT-11 was examined by means of the Mann-Whitney *U* test (Table 2). As a result, the expression levels of p21 and pRB showed significant differences that were associated with the IC_{50} values for CPT-11 (P=0.0339 and P=0.0109, respectively). In particular, the mean IC_{50} values of the low p21 expression group and the aberrant pRB group were 114.845 ng/ml and 128.560 ng/ml, respectively. These IC_{50} values are remarkably high, and, thus, CPT-11 is expected to be ineffective in cases showing a low expression of p21 and/or abnormal expression of pRB. These results suggest that the expression of p21 or pRB independently can be a good indicator of CPT-11 efficacy in ESCC.

Table 1. Relationship between percentages of total S-phase cells and cell proliferation activity values, and the expressions of cell cycle regulating proteins

	Percentage of total S-phase cells (mean±SD)	*P* value	Cell proliferation activity value (mean±SD)	*P* value
p53		0.6985		0.1213
Normal	28.688±11.606		2.410±0.245	
Overexpression	26.711±9.381		1.836±0.738	
Cyclin D1		0.2571		0.0719
Normal	25.266±9.788		1.866±0.696	
Overexpression	31.684±0.050		2.472±0.215	
p21		0.0477		0.0237
High	30.670±9.367		2.342±0.496	
Low	19.778±7.623		1.432±0.471	
p27		0.1556		0.1556
High	31.724±8.990		2.383±0.291	
Low	24.434±10.118		1.857±0.744	
pRB		0.0339		0.0047
Normal	30.719±9.266		2.426±0.340	
Abnormal	19.656±7.756		1.223±0.153	

Table 2. Relationship between IC_{50} values of CPT-11 and the expressions of cell cycle-regulating proteins

	IC_{50} value (mean±SD)	*P* value
p53		0.6985
Normal	37.200±29.072	
Overexpression	70.718±87.556	
Cyclin D1		0.6407
Normal	66.776±82.750	
Overexpression	37.592±32.486	
p21		0.0339
High	32.956±32.446	
Low	114.845±105.049	
p27		0.2453
High	32.123±31.996	
Low	74.525±84.901	
pRB		0.0109
Normal	27.470±25.633	
Abnormal	128.560±94.214	

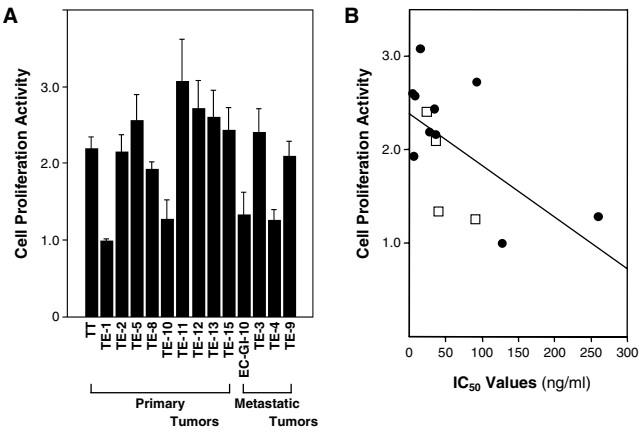

Fig. 4. Cell proliferation activity values of 14 ESCC cell lines measured by ELISA using BrdU (A). Cell proliferation activity values ranged from 0.999 to 3.083 with a mean±SD of 2.082±0.635. The mean cell proliferation activities of primary tumor cell lines (●) and metastatic tumor cell lines (□) were 2.203±0.648 and 1.780±0.565, respectively (B). The cell proliferation activity values of the 14 cell lines show an inverse correlation with the IC_{50} values for SN-38 (correlation coefficient=−0.601, *P*=0.0213).

Relationship between sensitivity to CPT-11 and cell proliferation activity. The cell proliferation activities of 14 ESCC cell lines *in vitro* were determined by ELISA using BrdU (Fig. 4A). The cell proliferation activity values ranged from 0.999 to 3.083 with a mean±SD of 2.082±0.635. The cell proliferation activity values of primary tumor cell lines and metastatic tumor cell lines were 2.203±0.648 and 1.780±0.565, respectively, and there was no significant difference between these 2 groups (Mann-Whitney *U* test).

A significant correlation was found between the cell proliferation activity values and the percentages of total S-phase cells (correlation coefficient=0.568, *P*=0.0324). Because BrdU is incorporated instead of thymidine into the DNA during DNA synthesis in proliferating cells, the cell proliferation activity using BrdU shows the ability to synthesize DNA, and reflects the percentage of total S-phase cells. As in the cell cycle analysis, among the 5 cell cycle-regulating factors, the expressions of p21 and pRB showed significant differences with cell proliferation activity values according to the Mann-Whitney *U* test (*P*=0.0237 and *P*=0.0047, respectively: Table 1). This suggests that ESCC cell lines showing low expression of p21 and/or aberrant expression of pRB have significantly poorer cell proliferation activities. Moreover, there was an inverse correlation observed between the IC_{50} values for CPT-11 and the cell proliferation activity values (correlation coefficient=−0.601, *P*=0.0213: Fig. 4B). This may indicate that high cell proliferation activity leads to an increased chance of ESCC cells entering S-phase, resulting in increased DNA damage by CPT-11; thus, cell proliferation activity could be an important indicator for sensitivity to CPT-11.

Discussion

In the previous study, we described the efficacy of CPT-11 for the treatment of ESCC, and suggested that the protein level of DNA topoisomerase I could be a predicting factor for the effect of CPT-11.[6] In the present study, we have found a significant relationship between the effect of CPT-11 and the percentage of S-phase cells. Following this result, we focused on cell cycle-regulating factors, and examined the relationship between the expression of individual cell cycle-regulating proteins and the therapeutic efficacy of CPT-11. As a result, the expressions of p21 and pRB were found to be predicting factors for sensitivity of ESCC to CPT-11. Namely, ESCC cell lines showing a low expression of p21 and/or aberrant expression of pRB were remarkably unresponsive to CPT-11 treatment.

Moreover, in this study, we examined cell proliferation activity using BrdU, and found a relationship with sensitivity to CPT-11. Because it has been reported that cell proliferation activity as determined using BrdU is closely related to the MIB-1 labeling index,[21–23] immunohistochemical studies of MIB-1 may make it possible to predict sensitivity to CPT-11 before treatment. Namely, our results suggest that immunohistochemical examinations of p21, pRB, and MIB-1, in addition to DNA topoisomerase I, in resected ESCC specimens and/or biopsy samples could be good indicators for sensitivity of ESCC patients to CPT-11.

CDDP is currently the key drug for the treatment of ESCC. However, its therapeutic efficacy is not satisfactory. Recently, CPT-11 has been reported to be effective for the treatment of many solid cancers, and has been selected as a second-line therapy. As for ESCC, CPT-11 is reported to be effective as a second-line chemotherapeutic agent when used with CDDP or docetaxel,[5, 24–26] and is a feasible option for use as a second-line chemotherapeutic agent. However, at the same time, CPT-11 causes severe adverse effects such as diarrhea, neutropenia, and nausea. ESCC patients with unresectable or relapsed tumors often develop poor performance status. Therefore, especially for second-line chemotherapy, it is important to predict the chemosensitivity of the cancer prior to treatment to avoid ineffective or harmful results. Our data suggest that examining the protein expressions of p21, pRB, and MIB-1 might be helpful for predicting the sensitivity of ESCC patients to CPT-11.

As for the relationship between ESCC chemosensitivity and cell cycle-regulating proteins, p53,[27, 28] cyclin D1,[29] p21,[30–32] and MIB-1[33] are reported to be effective markers. Many anticancer drugs, including CPT-11, demonstrate cytotoxicity by damaging DNA, and have S-phase-specific activity. Therefore, to examine the expressions of p21, pRB, and MIB-1 may help to predict tumor sensitivity to many anti-cancer agents, not only CPT-11.

Recently, various factors, such as p16ink4, NF kappaB, and MDM2, which regulate sensitivity to CPT-11, have been reported in many solid cancers.[34–36] In the future, it will be necessary to clarify the mechanism of the anticancer effect of CPT-11 and to look for more predicting factors for sensitivity to

CPT-11. This will help to identify enhancers of the therapeutic effect of CPT-11, and thus may lead to an improvement in the poor prognosis of ESCC patients.

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1. Iizuka T, Kakegawa T, Ide H, Ando N, Watanabe H, Tanaka O, Takagi I, Isono K, Ishida K, Arimori M. Phase II evaluation of cisplatin and 5-fluorouracil in advanced squamous cell carcinoma of the esophagus: a Japanese Esophageal Oncology Group Trial. *Jpn J Clin Oncol* 1992; **22**: 172–6.
2. Ando N, Ozawa S, Kitajima M, Iizuka T. Chemotherapy and multimodality therapy in the treatment of esophageal cancer. *Gan To Kagaku Ryoho* 1995; **22**: 869–76 (in Japanese).
3. Ilson DH, Forastiere A, Arquette M, Costa F, Heelan R, Huang Y, Kelsen DP. A phase II trial of paclitaxel and cisplatin in patients with advanced carcinoma of the esophagus. *Cancer J* 2000; **6**: 316–23.
4. Ajani JA. Docetaxel for gastric and esophageal carcinomas. *Oncology (Huntingt)* 2002; **16**: 89–96.
5. Ilson DH, Saltz L, Enzinger P, Huang Y, Kornblith A, O'Reilly E, Schwartz G, DeGroff J, Gonzalez G, Kelsen DP. Phase II trial of weekly irinotecan plus cisplatin in advanced esophageal cancer. *J Clin Oncol* 1999; **17**: 3270–5.
6. Nakajima Y, Miyake S, Nagai K, Kawano T, Iwai T. CPT-11 may provide therapeutic efficacy for esophageal squamous cell cancer and the effects correlate with the level of DNA topoisomerase I protein. *Jpn J Cancer Res* 2001; **92**: 1335–41.
7. Wang DY, Xiang YY, Tanaka M, Li XR, Li JL, Shen Q, Sugimura H, Kino I. High prevalence of p53 protein overexpression in patients with esophageal cancer in Linxian, China and its relationship to progression and prognosis. *Cancer* 1994; **74**: 3089–96.
8. Kawamura T, Goseki N, Koike M, Takizawa T, Endo M. Acceleration of proliferative activity of esophageal squamous cell carcinoma with invasion beyond the mucosa: immunohistochemical analysis of Ki-67 and p53 antigen in relation to histopathologic findings. *Cancer* 1996; **77**: 843–9.
9. Nakajima Y, Nagai K, Miyake S, Ohashi K, Kawano T, Iwai T. Evaluation of an indicator for lymph node metastasis of esophageal squamous cell carcinoma invading the submucosal layer. *Jpn J Cancer Res* 2002; **93**: 305–12.
10. Shinozaki H, Ozawa S, Ando N, Tsuruta H, Terada M, Ueda M, Kitajima M. Cyclin D1 amplification as a new predictive classification for squamous cell carcinoma of the esophagus, adding gene information. *Clin Cancer Res* 1996; **2**: 1155–61.
11. Sarbia M, Stahl M, Fink U, Heep H, Dutkowski P, Willers R, Seeber S, Gabbert HE. Prognostic significance of cyclin D1 in esophageal squamous cell carcinoma patients treated with surgery alone or combined therapy modalities. *Int J Cancer (Pred Oncol)* 1999; **84**: 86–91.
12. Sarbia M, Stahl M, zur Hausen A, Zimmermann K, Wang L, Fink U, Heep H, Dutkowski P, Willers R, Müller W, Seeber S, Gabbert HE. Expression of $p21^{WAF1}$ predicts outcome of esophageal cancer patients treated by surgery alone or by combined therapy modalities. *Clin Cancer Res* 1998; **4**: 2615–23.
13. Natsugoe S, Nakashima S, Matsumoto M, Xiangming C, Okumura H, Kijima F, Ishigami S, Takebayashi Y, Baba M, Takao S, Aikou T. Expression of $p21^{WAF1/Cip1}$ in the p53-dependent pathway is related to prognosis in patients with advanced esophageal carcinoma. *Clin Cancer Res* 1999; **5**: 2445–9.
14. Horwitz SB, Horwitz MS. Effects of camptothecin on the breakage and repair of DNA during the cell cycle. *Cancer Res* 1973; **33**: 2834–6.
15. Nishihira T, Hashimoto Y, Katayama M, Mori S, Kuroki T. Molecular and cellular features of esophageal cancer cells. *J Cancer Res Clin Oncol* 1993; **119**: 441–9.
16. Miyake S, Sellers WR, Safran M, Li X, Zhao W, Grossman SR, Gan J, DeCaprio JA, Adams PD, Kaelin WG Jr. Cells degrade a novel inhibitor of differentiation with E1A-like properties upon exiting the cell cycle. *Mol Cell Biol* 2000; **20**: 8889–902.
17. Kaye FJ, Kratzke RA, Gerster JL, Horowitz JM. A single amino acid substitution results in a retinoblastoma protein defective in phosphorylation and oncoprotein binding. *Proc Natl Acad Sci USA* 1990; **87**: 6922–6.
18. Templeton DJ, Park SH, Lanier L, Weinberg RA. Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. *Proc Natl Acad Sci USA* 1991; **88**: 3033–7.
19. Adams PD, Li X, Sellers WR, Baker KB, Leng X, Harper JW, Taya Y, Kaelin WG Jr. Rerinoblastoma protein contains a c-terminal motif that targets it for phosphorylation by cyclin-cdk complexes. *Mol Cell Biol* 1999; **19**: 1068–80.
20. Driscoll B, T'ang A, Hu YH, Yan CL, Fu Y, Luo Y, Wu KJ, Wen S, Shi XH, Barsky L, Weinberg K, Murphree AL, Fung YK. Discovery of a regulatory motif that controls the exposure of specific upstream cyclin-dependent kinase sites that determine both conformation and growth suppressing activity of pRB. *J Biol Chem* 1999; **274**: 9463–71.
21. Lynch DA, Clarke AM, Jackson P, Axon AT, Dixon MF, Quirke P. Comparison of labelling by bromodeoxyuridine, MIB-1, and proliferating cell nuclear antigen in gastric mucosal biopsy specimens. *J Clin Pathol* 1994; **47**: 122–5.
22. Onda K, Davis RL, Edwards MS. Comparison of bromodeoxyuridine uptake and MIB-1 immunoreactivity in medulloblastomas determined with single and double immunohistochemical staining methods. *J Neurooncol* 1996; **29**: 129–36.
23. Moriki T, Moriki T, Takahashi T, Kataoka H, Hiroi M, Yamane T, Hara H. Proliferation marker MIB-1 correlates well with proliferative activity evaluated by BrdU in breast cancer: an immunohistochemical study including correlation with PCNA, p53, c-erbB-2 and estrogen receptor status. *Pathol Int* 1996; **46**: 953–61.
24. Ajani JA, Faust J, Yao J, Komaki R, Stevens C, Swisher S, Putnam JB, Vaporciyan A, Smythe R, Walsh G, Rice D, Roth J. Irinotecan/cisplatin followed by 5-FU/paclitaxel/radiotherapy and surgery in esophageal cancer. *Oncology (Huntingt)* 2003; **17**: 20–2.
25. Lordick F, von Schilling C, Bernhard H, Hennig M, Bredenkamp R, Peschel C. Phase II trial of irinotecan plus docetaxel in cisplatin-pretreated relapsed or refractory oesophageal cancer. *Br J Cancer* 2003; **89**: 630–3.
26. Govindan R, Read W, Faust J, Trinkaus K, Ma MK, Baker SD, McLeod HL, Perry MC. Phase II study of docetaxel and irinotecan in metastatic or recurrent esophageal cancer: a preliminary report. *Oncology (Huntingt)* 2003; **17**: 27–31.
27. Puglisi F, Di Loreto C, Panizzo R, Avellini C, Fongione S, Cacitti V, Beltrami CA. Expression of p53 and bcl-2 and response to preoperative chemotherapy and radiotherapy for locally advanced squamous cell carcinoma of the esophagus. *J Clin Pathol* 1996; **49**: 456–9.
28. Krasna MJ, Mao YS, Sonett JR, Tamura G, Jones R, Suntharalingam M, Meltzer SJ. P53 gene protein overexpression predicts results of trimodality therapy in eshophageal cancer patients. *Ann Thorac Surg* 1999; **68**: 2021–4.
29. Samejima R, Kitajima Y, Yunotani S, Miyazaki K. Cyclin D1 is a possible predictor of sensitivity to chemoradiotherapy for esophageal squamous cell carcinoma. *Anticancer Res* 1999; **19**: 5515–21.
30. Shimoyama S, Konishi T, Kawahara M, Aoki F, Harada N, Shimizu S, Murakami T, Kaminishi M. Expression and alteration of p53 and p21(waf1/cip1) influence the sensitivity of chemoradiation therapy for esophageal cancer. *Hepatogastroenterology* 1998; **45**: 1497–504.
31. Kuwahara M, Hirai T, Yoshida K, Yamashita Y, Hihara J, Inoue H, Toge T. p53, p21(Waf1/Cip1) and cyclin D1 protein expression and prognosis in esophageal cancer. *Dis Esophagus* 1999; **12**: 116–9.
32. Nakashima S, Natugoe S, Matsumoto M, Kijima F, Takebayashi Y, Okumura H, Shimada M, Nakano S, Baba M, Takao S, Aikou T. Expression of p53 and p21 is useful for the prediction of preoperative chemotherapeutic effects in eshophageal carcinoma. *Anticancer Res* 2000; **20**: 1933–7.
33. Kitamura K, Saeki H, Kawaguchi H, Araki K, Ohno S, Kuwano H, Maehara Y, Sugimachi K. Immunohistochemical status of the p53 protein and Ki-67 antigen using biopsied specimens can predict a sensitivity to neoadjvant therapy in patients with esophageal cancer. *Hepatogastroenterology* 2000; **47**: 419–23.
34. Fukuoka K, Nishio K, Fukumoto H, Arioka H, Kurokawa H, Ishida T, Iwamoto Y, Tomonari A, Suzuki T, Ushida J, Narita N, Saijo N. Ectopic p16(ink4) expression enhances CPT-11-induced apoptosis through increased delay in S-phase progression in human non-small-cell-lung-cancer cells. *Int J Cancer* 2000; **86**: 197–203.
35. Cusack JC Jr, Liu R, Houston M, Abendroth K, Elliott PJ, Adams J, Baldwin AS Jr. Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-kappaB inhibition. *Cancer Res* 2001; **61**: 3535–40.
36. Wang H, Yu D, Agrawal S, Zhang R. Experimental therapy of human prostate cancer by inhibiting MDM2 expression with novel mixed-backbone antisense oligonucleotides: *in vitro* and *in vivo* activities and mechanisms. *Prostate* 2003; **54**: 194–205.