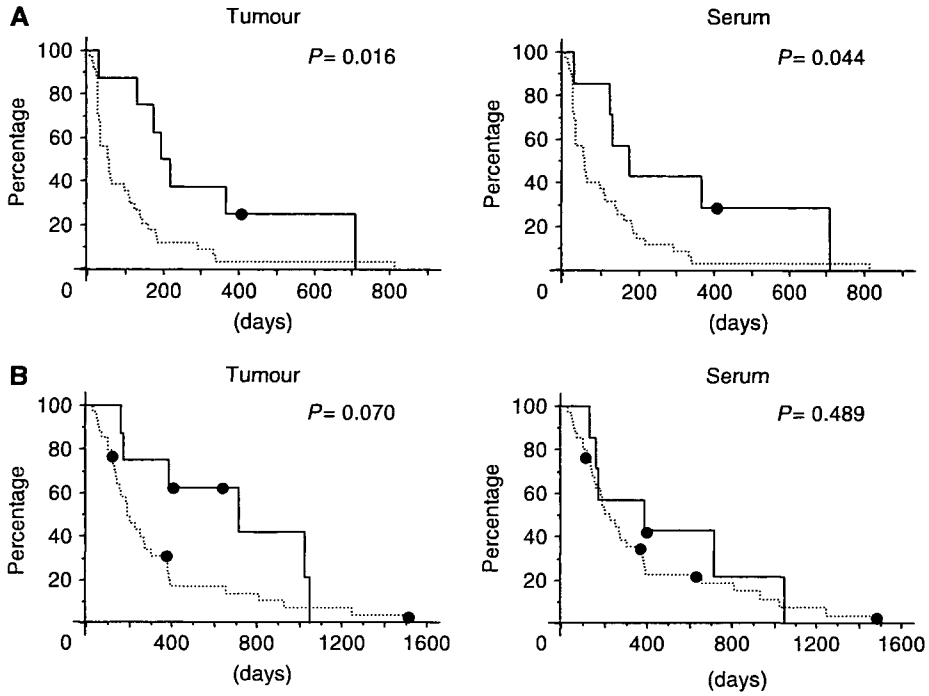

Figure 1 Kaplan–Meier probability of progression-free survival (**A**) and overall survival (**B**) with respect to the EGFR mutation status of NSCLC. *P*-values were calculated by the log-rank test.

reliability of *EGFR* mutation detection in serum DNA as a predictive marker of response to gefitinib.

The sites of the *EGFR* mutations detected in this study are identical to those reported in previous studies (Kosaka *et al*, 2004; Pao *et al*, 2004). The majority mutations were in-frame deletions in exon 19 and the missense mutation L858R in exon 21. The comparison between mutation status and clinical manifestations in this study confirmed the finding in previous studies that *EGFR* mutations are frequently present in small subgroups of NSCLC patients, including females, never-smokers, and patients with adenocarcinoma histology, although these findings were not statistically significant.

EGFR mutations were detected in only 1.0 ml serum samples. The amount of DNA extracted was minute, and its concentration in roughly one-third of patients was below the minimum concentration detectable by spectrophotometry. Moreover, lung cancers are very heterogeneous, and patients' serum also contains DNA derived from normal cells. Direct sequencing seems unable to provide satisfactory results for detection of *EGFR* mutations in samples containing a mixture of mutated and wild-type DNA. Although direct sequencing has generally been used to detect *EGFR* mutations, detection by direct sequencing requires at least 30% of the DNA in the sample to be mutated (Bosari *et al*, 1995; Fan *et al*, 2001). Small amounts and low percentages of mutated DNA in serum can be missed by direct sequencings. When serum is used as the material for detection of *EGFR* mutations, patients with *EGFR* mutations may be diagnosed as having wild-type *EGFR* because of the two limitations described above. In this study, the mutation was detected by direct sequencing in only one patient. The mutation status detected by Scorpion ARMS in serum samples was nearly identical to that in tumour samples. The concentrations of serum DNA in two of seven patients with *EGFR* mutations in serum samples were below the minimum concentration detectable. The high-sensitive method, Scorpion ARMS, completely resolved the problem.

The mutation status in the pairs of samples from three patients (3 out of 42, 7.1%) did not match. The results in the serum DNA of two patients were mutation-negative, whereas mutations were detected in actual tumour samples. The amount of tumour-specific DNA may have been below the threshold of detection with the Scorpion ARMS Kit in the patient with L858R. Little tumour-specific DNA may be circulating in patients, and the quality of the DNA is also a determinant of successful detection. Prolonged storage of serum samples has been reported to result in a decrease in the amount of DNA extracted (Sozzi *et al*, 2005). The other patient had an E746_T751del, and the mutation was not detected with the Scorpion ARMS in the patients. Although we have showed the usefulness of Scorpion ARMS for detection of *EGFR* mutation in serum samples (Kimura *et al*, 2006), Scorpion ARMS is only able to detect mutations targeted by the Scorpion primers designed in advance and in this study was capable of detecting the specific mutation of E746_A750del in exon 19 and L858R in exon 21. E747_P753del insS and L747_T751del are minor variations of deletional mutations in exon 19 and were not detected by this method in a preliminary experiment (data not shown). We do not think that E746_T751del can be detected with Scorpion ARMS. Mutation status in serum DNA was positive (V689L and L858R) in one patient in whom no mutations were detected in actual tumour samples. V689L and L858R are somatic mutations. We concluded that the direct sequencing of DNA from the tumour sample yielded the wrong result. Low rate of tumour-derived DNA in total DNA or impure DNA extracted from tumour samples may have prevented a detection of the mutation by direct sequencing.

On the basis of the results of this study, we conclude that it is feasible to use serum DNA to detect *EGFR* mutation status and evaluate its potential as a predictor of response to EGFR-TKI. The serum assay to detect *EGFR* mutations circumvents the need for tumour tissue and merits further validation of the use of serum DNA to detect *EGFR* mutations as a predictor of response to, and survival on gefitinib in prospective studies.

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Synergistic antitumor activity of the novel SN-38-incorporating polymeric micelles, NK012, combined with 5-fluorouracil in a mouse model of colorectal cancer, as compared with that of irinotecan plus 5-fluorouracil

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The authors reported in a previous study that NK012, a 7-ethyl-10-hydroxy-camptothecin (SN-38)-releasing nano-system, exhibited high antitumor activity against human colorectal cancer xenografts. This study was conducted to investigate the advantages of NK012 over irinotecan hydrochloride (CPT-11) administered in combination with 5-fluorouracil (5FU). The cytotoxic effects of NK012 or SN-38 (an active metabolite of CPT-11) administered in combination with 5FU was evaluated *in vitro* in the human colorectal cancer cell line HT-29 by the combination index method. The effects of the same drug combinations was also evaluated *in vivo* using mice bearing HT-29 and HCT-116 cells. All the drugs were administered i.v. 3 times a week; NK012 (10 mg/kg) or CPT11 (50 mg/kg) was given 24 hr before 5FU (50 mg/kg). Cell cycle analysis in the HT-29 tumors administered NK012 or CPT-11 *in vivo* was performed by flow cytometry. NK012 exerted more synergistic activity with 5FU compared to SN-38. The therapeutic effect of NK012/5FU was significantly superior to that of CPT-11/5FU against HT-29 tumors ($p = 0.0004$), whereas no significant difference in the antitumor effect against HCT-116 tumors was observed between the 2-drug combinations ($p = 0.2230$). Cell-cycle analysis showed that both NK012 and CPT-11 tend to cause accumulation of cells in the S phase, although this effect was more pronounced and maintained for a more prolonged period with NK012 than with CPT-11. Optimal therapeutic synergy was observed between NK012 and 5FU, therefore, this regimen is considered to hold promise of clinical benefit, especially for patients with colorectal cancer.

Key words: NK012; SN-38; 5-fluorouracil; drug delivery system; colorectal cancer

The 5-year survival rates of colorectal cancer (CRC) have improved remarkably over the last 10 years, accounted for in large part by the extensively investigated agents after 5-fluorouracil (5FU). Irinoetcan hydrochloride (CPT-11), a water-soluble, semi-synthetic derivative of camptothecin, is one such agent that has been shown to be highly effective, and currently represents a key-drug in first- and second-line treatment regimens for CRC. CPT-11 monotherapy, however, has not been shown to yield superior efficacy, including in terms of the median survival time, to bolus 5FU/leucovorin (LV) alone.[1] In 2 Phase III trials, the addition of CPT-11 to bolus or infusional 5FU/LV regimens clearly yielded greater efficacy than administration of 5FU/LV alone, with a doubling of the tumor response rate and prolongation of the median survival time by 2–3 months.[1,2]

CPT-11 is converted to 7-ethyl-10-hydroxy-camptothecin (SN-38), a biologically active and water-insoluble metabolite of CPT-11, by carboxylesterases in the liver and the tumor. SN-38 has been demonstrated to exhibit up to a 1,000-fold more potent cytotoxic activity than CPT-11 against various cancer cells *in vitro*.[3] The metabolic conversion rate is, however, very low, with only <10% of the original volume of CPT-11 being metabolized to SN-38[4,5]; conversion of CPT-11 to SN-38 also depends on genetic interindividual variability of the activity of carboxylesterases.[6] Direct use of SN-38 itself for clinical cancer treatment must be shown to be identical in terms of both efficacy and toxicity.

Some drugs incorporated in drug delivery systems (DDS), such as Abraxane and Doxil, are already in clinical use.[7,8] The clinical benefits of DDS are based on their EPR effect.[9] The EPR effect is based on the pathophysiological characteristics of solid tumor tissues: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from the tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. Several types of DDS can be used for incorporation of a drug. A liposome-based formulation of SN-38 (LE-SN38) has been developed, and a clinical trial to assess its efficacy is now under way.[10,11]

Recently, we demonstrated that NK012, novel SN-38-incorporating polymeric micelles, exerted superior antitumor activity and less toxicity than CPT-11.[12] NK012 is characterized by a smaller size of the particles than LE-SN38; the mean particle diameter of NK012 is 20 nm. NK012 can release SN-38 under neutral conditions even in the absence of a hydrolytic enzyme, because the bond between SN-38 and the block copolymer is a phenol ester bond, which is stable under acidic conditions and labile under mild alkaline conditions. The release rate of SN-38 from NK012 under physiological conditions is quite high; more than 70% of SN-38 is released within 48 hr. We speculated that the use of NK012, in place of CPT-11, in combination with 5FU may yield superior results in the treatment of CRC. In the present study, we evaluated the antitumor activity of NK012 administered in combination with 5FU as compared to that of CPT-11 administered in combination with 5FU against CRC in an experimental model.

Material and methods

Cells and animals

The human colorectal cancer cell lines used, namely, HT-29 and HCT-116, were purchased from the American Type Culture Collection (Rockville, MD). The HT-29 cells and HCT-116 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gaggenau-Hoerden, Germany), penicillin, streptomycin, and amphotericin B (100 units/mL, 100 μg/mL, and 25 μg/mL, respectively; Sigma, St. Louis, MO) in a humidified atmosphere containing 5% CO_2 at 37°C.

BALB/c *nu/nu* mice were purchased from SLC Japan (Shizuoka, Japan). Six-week-old mice were subcutaneously (s.c.)

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inoculated with 1×10^6 cells of HT-29 or HCT-116 cell line in the flank region. The length (a) and width (b) of the tumor masses were measured twice a week, and the tumor volume (TV) was calculated as follows: $TV = (a \times b^2)/2$. All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of the National Cancer Center; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Drugs

The SN-38-incorporating polymeric micelles, NK012, and SN-38 were prepared by Nippon Kayaku (Tokyo, Japan).[12] CPT-11 was purchased from Yakult Honsha (Tokyo, Japan). 5FU was purchased from Kyowa Hakko (Tokyo, Japan).

Cell growth inhibition assay

HT-29 cells were seeded in 96-well plates at a density of 2,000 cells/well in a final volume of 90 μL. Twenty-four hours after seeding, a graded concentration of NK012 or SN-38 was added concurrently with 5FU to the culture medium of the HT-29 cells in a final volume of 100 μL for drug interaction studies. The culture was maintained in the CO_2 incubator for an additional 72 hr. Then, cell growth inhibition was measured by the tetrazolium salt-based proliferation assay (WST assay; Wako Chemicals, Osaka, Japan). WST-1 labeling solution (10 μL) was added to each well and the plates were incubated at 37°C for 3 hr. The absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader. Cell viability was measured and compared to that of the control cells. Each experiment was carried out in triplicate and was repeated at least 3 times. Data were averaged and normalized against the nontreated controls to generate dose-response curves.

Drug interaction analysis

The nature of interaction between NK012 or SN-38 and 5FU against HT-29 cells was evaluated by median-effect plot analyses and the combination index (CI) method of Chou and Talalay.[13] Data analysis was performed using the Calcusyn software (Biosoft, NY, USA). NK012 or SN-38 was combined with 5FU at a fixed ratio that spanned the individual IC_{50} values of each drug. The IC_{50} values were determined on the basis of the dose-response curves using the WST assay. For any given drug combination, the CI is known to represent the degree of synergy, additivity or antagonism. It is expressed in terms of fraction-affected (Fa) values, which represents the percentage of cells killed or inhibited by the drug. Isobologram equations and Fa/CI plots were constructed by computer analysis of the data generated from the median effect analysis. Each experiment was performed in triplicate with 6 gradations and was repeated at least 3 times. The resultant dose-response curves were averaged, to create a single composite dose-response curve for each combination.

In vivo analysis of the effects of NK012 combined with 5FU as compared to those of CPT-11 combined with 5FU

When the mean tumor volumes reached ~93 mm^3, the mice were randomly divided into test groups consisting of 5 mice per group (Day 0). The drugs were administered i.v. *via* the tail vein of the mice. In the groups administered NK012 or 5FU as single agents, the drug was administered on Days 0, 7 and 14. In the combined treatment groups, NK012 or CPT-11 was administered 24 hr before 5FU on Days 0, 7 and 14, according to the previously reported combination schedule for CPT-11 and 5FU.[14] Complete response (CR) was defined as tumor not detectable by palpation at 90 days after the start of treatment, at which time-point the mice were sacrificed. Tumor volume and body weight were measured twice a week. As a general rule, animals in which the tumor volume exceeded 2,000 mm^3 were also sacrificed.

Experiment 1. Evaluation of the effects of NK012 combined with 5FU and determination of the maximum tolerated dose (MTD) of NK012/5FU. By comparing the data between NK012 administered as a single agent and NK012/5FU, we evaluated the effects of the combined regimen against the s.c HT-29 tumors. A preliminary experiment showed that combined administration of NK012 15 mg/kg + 5FU 50 mg/kg every 6 days caused drug-related lethality (data not shown). To determine the MTD, therefore, we set the dosing schedule of the combined regimen at 5 or 10 mg/kg of NK012 + 50 mg/kg of 5FU three times a week.

Experiment 2. Comparison of the antitumor effect of NK012/5FU and CPT-11/5FU. Based on a comparison of the data between NK012/5FU and CPT-11/5FU against the s.c. HT-29 and HCT-116 tumors, we investigated the feasibility of the clinical application of NK012/5FU for the treatment of CRC. CPT-11/5FU was administered three times a week at the respective MTDs of the 2 drugs as previously reported, that is, CPT11 at 50 mg/kg and 5FU at 50 mg/kg, respectively.[14] NK012/5FU was administered once three times a week at the respective MTDs of the 2 drugs determined from Experiment 1.

Cell cycle analysis

Samples from the HT-29 tumors that had grown to 80–100 mm^3 were removed from the mice at 6, 24, 48, 72 and 96 hr after the administration of NK012 alone at 10 mg/kg or CPT-11 alone at 50 mg/kg. The samples were excised, minced in PBS and fixed in 70% ethanol at −20°C for 48 hr. They were then digested with 0.04% pepsin (Sigma chemical Co., St Lous, MO) in 0.1 N HCL for 60 min at 37°C in a shaking bath to prepare single-nuclei suspensions. The nuclei were then centrifuged, washed twice with PBS and stained with 40 μg/mL of propidium iodide (Molecular Probes, OR) in the presence of 100 μg/mL RNase in 1 mL PBS for 30 min at 37°C. The stained nuclei were analyzed with B-D FACSCalibur (BD Biosciences, San Jose, CA), and the cell cycle distribution was analyzed using the Modfit program (Verity Software House Topsham, ME).

Statistical analyses

Data were expressed as mean ± SD. Data were analysed with Student's t test when the groups showed equal variances (F test), or Welch's test when they showed unequal variances (F test). $p < 0.05$ was regarded as statistically significant. All statistical tests were 2-sided.

Results

Antiproliferative effects of NK012 or SN-38 administered in combination with 5FU

Figure 1*a* shows the dose-response curves for NK012 alone, 5FU alone and a combination of the two. The IC_{50} levels of NK012 and 5FU against the HT-29 cells were 39 nM and 1 μM, respectively, and the IC_{50} level of SN-38 was 14 nM (data not shown). Based on these data, the molar ratio of NK012 or SN-38:5FU of 1:1,000 was used for the drug combination studies.

Figures 1*b* and 1*c* show the median-effect and the combination index plots. Combination indices (CIs) of <1.0 are indicative of synergistic interactions between 2 agents; additive interactions are indicated by CIs of 1.0, and antagonism by CIs of >1.0. Figure 1*c* shows the combination index for NK012 and 5FU, when 2 drugs are supposed to be mutually exclusive. Marked synergism was observed between Fa 0.2 and 0.6. Theoretically, the CI method is the most reliable around an Fa of 0.5, suggesting synergistic effects of the combination of NK012 and 5FU. This synergistic effect was more evident than that of SN-38/5FU (Fig. 1*d*).

In vivo *effect of combined NK012 and 5FU*

Experiment 1. Dose optimization and effect of combined NK012 and 5FU against HT-29 tumors. Comparison of the relative tumor volumes on Day 40 revealed significant differences between

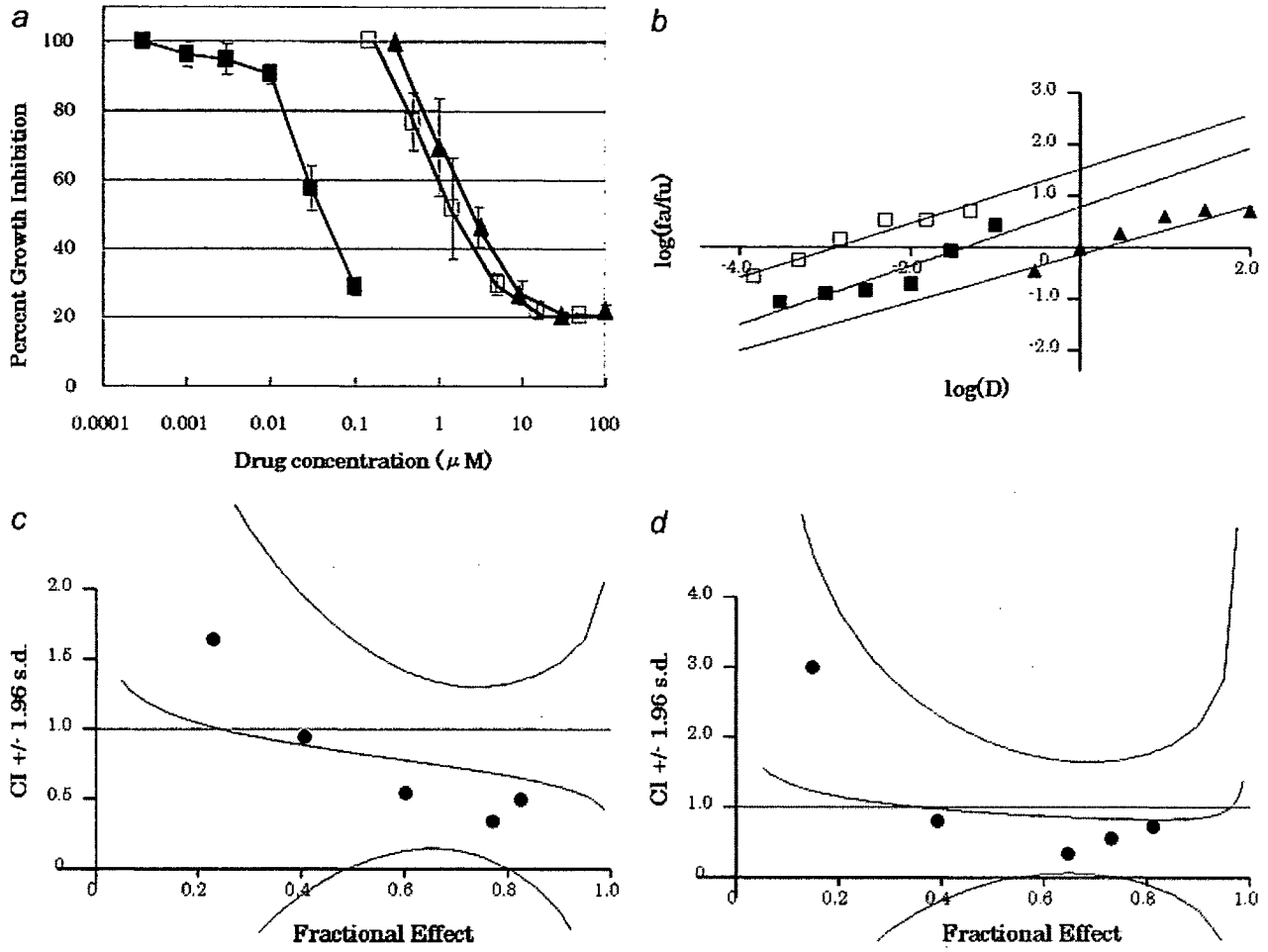


FIGURE 1 – Interaction of NK012 and 5FU *in vitro*. (*a*) Dose-response curves for NK012 alone (■), 5FU alone (▲) and their combination (□) against HT-29 cells. HT-29 cells were seeded at 2,000 cells/well. Twenty-four hours after seeding, a graded concentration of NK012 or 5FU was added to the culture medium of the HT-29 cells. Cell growth inhibition was measured by WST assay after 72 hr of treatment. Cell viability was measured and compared with that of the control cells. Each experiment was carried out independently and repeated at least 3 times. Points, mean of tripricates; bars, SD. (*b*) Median effect plot for the interaction of NK012 and 5FU. (*c*, *d*) Combination index for the interaction as a function of the level of effect (fraction effect = 0.5 is the IC_{50}). The straight line across the CI value of 1.0 indicates additive effect and CIs above and below indicate antagonism and synergism, respectively. The molar ratio of NK012/5FU (*c*) or SN-38/5FU (*d*) at 1:1,000 was tested by CI analysis. Black circles represent the CIs of the actual data points, solid lines represent the computer-derived CIs at effect levels ranging from 10 to 100% inhibition of cell growth, and the dotted lines represent the 95% confidence intervals.

those in the mice administered NK012 alone and those administered NK012/5FU at 5 mg/kg of NK012 ($p = 0.018$) (Fig. 2*a*). Although there was no statistically significant difference in the relative tumor volume measured on Day 54 between the mice administered NK012 alone and NK012/5FU at 10 mg/kg of NK012 ($p = 0.3050$), a trend of superior antitumor effect was demonstrated in the group treated with NK012/5FU at 10 mg/kg of NK012 (Fig. 2*a*). The CR rates were 20, 40 and 60% for 5 mg/kg NK012 + 50 mg/kg 5FU, 10 mg/kg NK012 alone and 10 mg/kg NK012 + 50 mg/kg 5FU, respectively. The schedule of 10 mg/kg NK012 + 50 mg/kg 5FU resulted in no remarkable toxicity in terms of body weight changes, and these doses were determined as representing the MTDs (Fig. 2*b*).

Experiment 2. Comparison of the antitumor effect of combined NK012/5FU and CPT-11/5FU against HT-29 and HCT-116 tumors. The therapeutic effect of NK012/5FU on Day 60 was significantly superior to that of CPT-11/5FU against the HT-29 tumors ($p = 0.0004$) (Fig. 3*a*). A more potent antitumor effect, namely, a 100% CR rate, was obtained in the NK012/5FU group as compared to the 0% CR rate in the CPT-11/5FU group. Although no statistically significant difference in the relative tumor volume on Day 61 was demonstrated between the NK012/5FU and CPT-11/5FU in the case of the HCT-116 tumors ($p = 0.2230$), a trend of superior antitumor effect against these tumors was observed in the NK012/5FU treatment group (Fig. 3*b*). The CR rates for the case of the HCT-116 tumors were 0% in both NK012/5FU and CPT-11/5FU groups.

Specificity of cell cycle perturbation

We studied the differences in the effects between NK012 10 mg/kg and CPT-11 50 mg/kg on the cell cycle (Fig. 4*a*). The data indicated that both NK012 and CPT-11 tended to cause accumulation of cells in the S phase, although the effect of NK012 was stronger and maintained for a more prolonged period than that of CPT-11; the maximal percentage of S-phase cells in the total cell population in the tumors was 34% at 24 hr after the administration of CPT-11, whereas it was 39% at 48 hr after the administration of NK012 (Figs. 4*b*, and 4*c*).

Discussion

Our primary endpoint was to clarify the advantages of NK012 over CPT-11 administered in combination with 5FU. We demonstrated that combined NK012 and 5FU chemotherapy exerts more

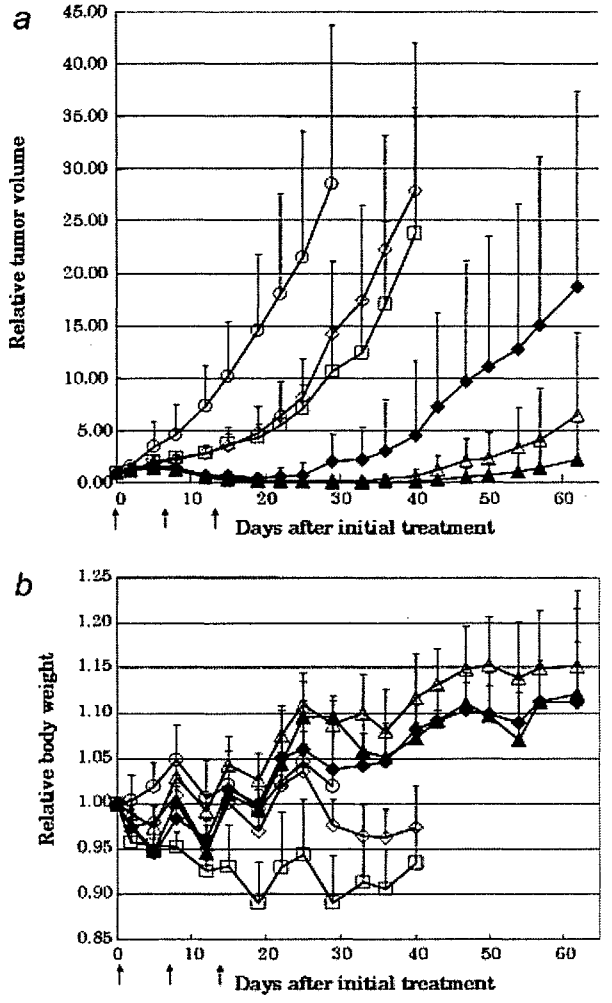


FIGURE 2 – Effect of NK012 alone or NK012 in combination with 5FU against HT-29 tumor-bearing mice. Points, mean; bars, SD. (*a*) Antitumor effect of each regimen on Days 0, 7 and 14. (○) control, (□) 5FU 50 mg/kg alone, (◇) NK012 5 mg/kg alone, (◆) NK012 5 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg alone, (▲) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. (*b*) Changes in the relative body weight. Data were derived from the same mice as those used in the present study.

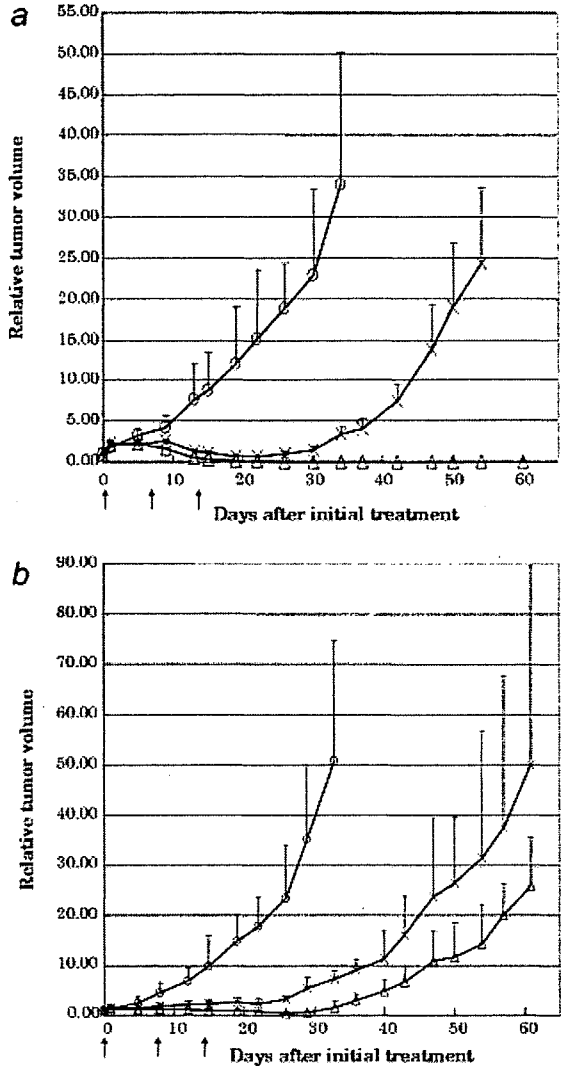
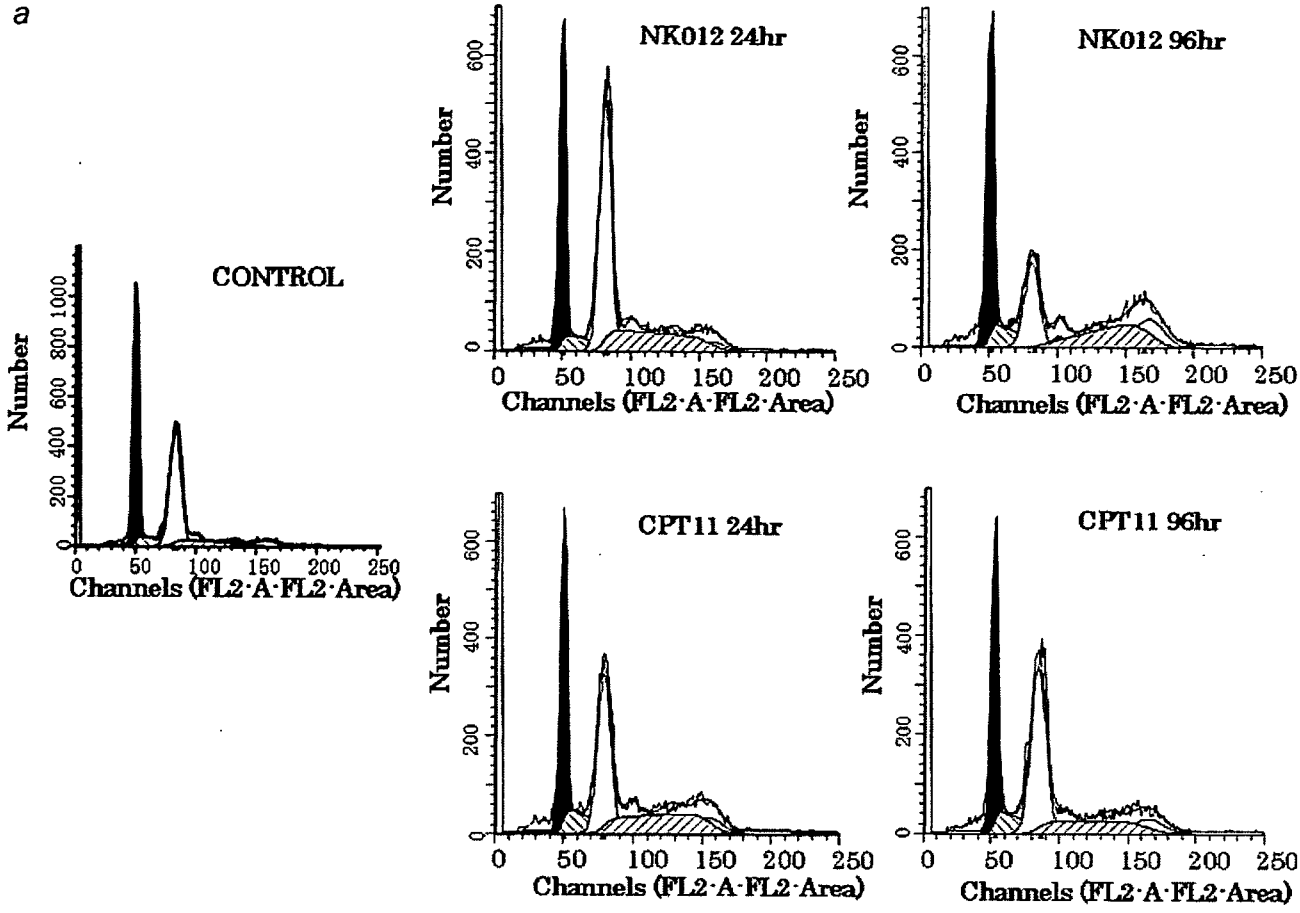


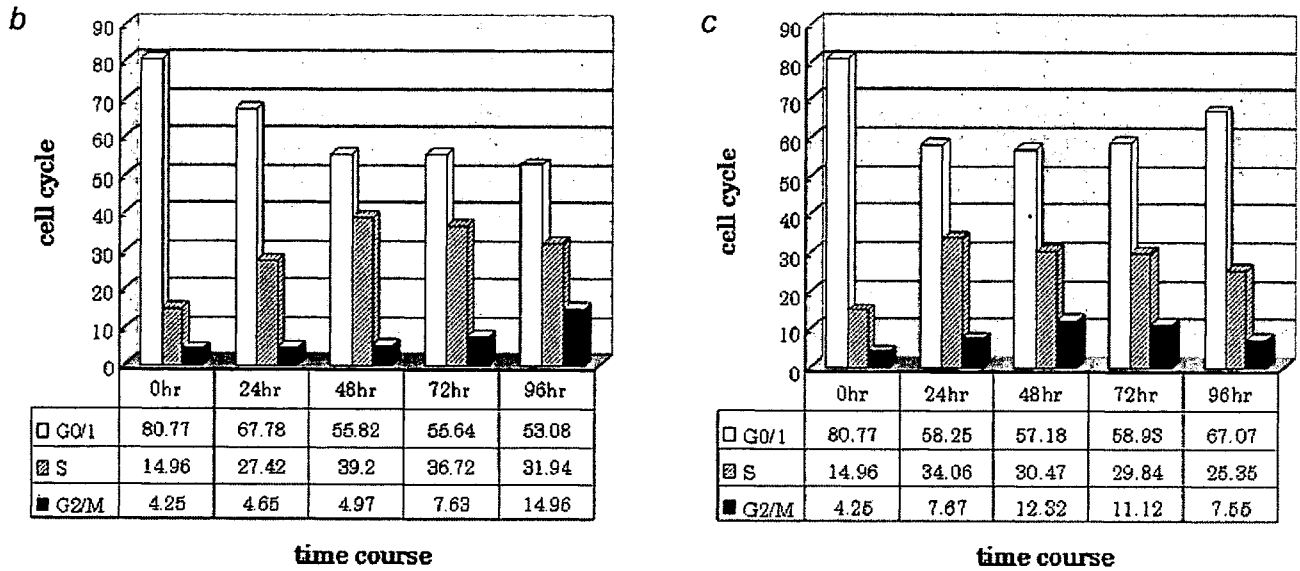
FIGURE 3 – Effect of NK012/5FU as compared with that of CPT11/5FU against HT-29 (*a*) or HCT-116 (*b*) tumor-bearing mice. Antitumor effect of each schedule on Days 0, 7 and 14. (○) control, (×) CPT-11 50 mg/kg 24 hr before 5FU 50 mg/kg, (△) NK012 10 mg/kg 24 hr before 5FU 50 mg/kg. Points, mean; bars, SD.

synergistic activity *in vitro* and significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU. The combination of NK012 and 5FU is considered to hold promise of clinical benefit for patients with CRC.

CPT-11, a topoisomerase-I inhibitor, and 5FU, a thymidilate synthase inhibitor, have been demonstrated to be effective agents for the treatment of CRC. A combination of these 2 drugs has also been demonstrated to be clearly more effective than either CPT-11 or 5FU/LV administered alone *in vivo* and in clinical settings.[1,2,14] Administration of 5FU by infusion with CPT-11 was shown to be associated with reduced toxicity and an apparent improvement in survival as compared to that of administration of the drug by bolus injection with CPT-11.[1,2] This synergistic enhancement may result from the mechanism of action of the 2 drugs; CPT-11 has been reported to cause accumulation of cells in the S phase, and 5FU infusion is known to cause DNA damage specifically in cells of the S phase.[14] On the basis of this background, our results suggesting the more pronounced and more prolonged accumulation of the tumor cells in the S phase caused by NK012 as compared with that by CPT-11 may explain the more effective synergy of the former administered with 5FU infusion. This may be attributable to accumulation of NK012 due to the enhanced permeability and retention (EPR) effect.[9] It is also speculated that NK012 allows sustained release of free SN-38, which may move more freely in the tumor interstitium.[15] Otherwise NK012 itself could internalize into cells to localize in several cytoplasmic organelles as reported by Savic *et al.*[16] These characteristics of NK012 may be responsible for its more potent antitumor activity observed in this study, because CPT-11 has been reported to show time-dependent growth-inhibitory activity against the tumor cells.[17]

The major dose-limiting toxicities of CPT-11 are diarrhea and neutropenia. SN-38, the active metabolite of CPT-11, may cause CPT-11-related diarrhea as a result of mitotic -inhibitory activity.[18] Because it undergoes significant biliary excretion, SN-38 may have a potentially long residence time in the gastrointestinal tract that may be associated with prolonged diarrhea.[19,20] In our previous report, we evaluated the tissue distribution of SN-38 after administration of an equimolar amount of NK012 (20 mg/kg) and CPT-11 (30 mg/kg), and found no difference in the level of SN-38 accumulation in the small intestine.[12] A significant antitumor effect of NK012 with a lower incidence of diarrhea was also dem-





	0hr	24hr	48hr	72hr	96hr
G0/1	80.77	67.78	55.82	55.64	53.08
S	14.96	27.42	39.2	36.72	31.94
G2/M	4.25	4.65	4.97	7.63	14.96

	0hr	24hr	48hr	72hr	96hr
G0/1	80.77	58.25	57.18	58.93	67.07
S	14.96	34.06	30.47	29.84	25.35
G2/M	4.25	7.67	12.32	11.12	7.55

FIGURE 4 – Cell cycle analysis of HT-29 tumor cells collected 24, 48, 72 and 96 hr after administration of NK012 at 10 mg/kg alone or CPT-11 at 50 mg/kg alone using the Modfit program (Verity Software House Topsham, ME). (*a*) Cell cycle analysis of HT-29 tumor cells 24 and 96 hr after administration of NK012 at 10 mg/kg or CPT-11 at 50 mg/kg, respectively. (*b*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with NK012 at 10 mg/kg. (*c*) Cell cycle distribution of tumor cells 0, 24, 48, 72 and 96 hr after treatment with CPT-11 at 50 mg/kg.

onstrated as compared to that observed with CPT-11 in a rat mammary tumor model.[21] Combined administration of CPT-11 with 5FU/LV infusion appears to be associated with acceptable toxicity in patients with CRC. In addition, no significant difference in the frequency of Grade 3/4 diarrhea was noted between patients treated with FOLFIRI (CPT-11 regimen with bolus and infusional 5FU/LV) and those treated with FOLFOX6 (oxaliplatin regimen with bolus and infusional 5FU/LV).[22,23] Our *in vivo* data actually revealed no severe body weight loss in the NK012/5FU group. Consequently, we expect that the NK012/5FU regimen, especially

with infusional 5FU, may be an attractive arm for a Phase III trial in CRC, with CPT-11/5FU as the control arm. We have already initiated a Phase I trial of NK012 in patients with advanced solid tumors based on the data suggesting higher efficacy and lower toxicity of this preparation than CPT-11 *in vivo*.[12]

In conclusion, we demonstrated that combined NK012 and 5FU chemotherapy exerts significantly greater antitumor activity against human CRC xenografts as compared to CPT-11/5FU, indicating the necessity of clinical evaluation of this combined regimen.

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Expression of beta-tubulin isotypes in human primary ovarian carcinoma

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Abstract

Objective. Selective expression of beta-tubulin isotypes has been reported to be one of the important mechanisms of taxane resistance. The purpose of this study was to evaluate the immunohistochemical expression of beta-tubulin isotypes using clinical samples of ovarian carcinoma treated by taxanes and to examine whether the protein levels of each of the beta-tubulin isotypes were correlated with the clinical features.

Experimental design. We examined tumor samples taken from 77 ovarian carcinoma patients (54 patients treated with a taxane-based regimen and 23 treated with a taxane-free regimen), for the intrinsic protein level of beta-tubulin isotype (classes I, II, III and IV) expression using immunohistochemistry, and we evaluated the correlation of this protein level with the clinical features. The expression levels were scored by the proportion and intensity of the immunoreactive tumor cells.

Results. High protein levels of classes I and IV beta-tubulin, and very low protein levels of class II beta-tubulin, and intermediate protein levels of class III beta-tubulin expression were demonstrated in a total of 77 ovarian carcinomas. As for the samples taken from the 54 patients treated with the taxane-based regimen, 40 samples demonstrated undetectable levels of class II beta-tubulin protein. The class II beta-tubulin expression-absent group was significantly correlated with advanced stage ($p=0.024$) and with a short period of progression-free survival (log-rank test, $p=0.022$). Multivariate analyses demonstrated that the only significant independent prognostic indicator of a short period of progression-free survival was advanced stage, although a high expression of class III beta-tubulin was also prone to be associated with a short period of progression-free survival, but not significantly so ($p=0.081$). No such correlations or propensities were demonstrated in the 23 patients treated with the taxane-free regimen.

Conclusions. In cases of ovarian carcinoma treated by taxanes, high expression of class III beta-tubulin seems to be associated with earlier recurrence, which is believed likely to be resistant relapse. In addition, loss of class II beta-tubulin expression is correlated with advanced stage, which may represent aggressive tumor progression.

Keywords: Beta-tubulin isotypes; Immunohistochemistry; Taxane; Ovarian cancer

Introduction

Patients with advanced ovarian carcinoma have been treated by debulking surgery followed by chemotherapy containing taxanes. Taxane-based combination chemotherapy plays a major role in the treatment of ovarian carcinoma. Although the majority of advanced ovarian carcinomas will initially respond to chemotherapy, most will relapse and fail to respond successfully to further treatments. Resistance to anticancer drugs especially taxanes presents a major obstacle in attempts to improve clinical outcome.

One of the important mechanisms of taxane resistance is a decrease in the accumulation of the drug, caused by enhanced drug efflux, mediated by transporters such as P-glycoprotein (P-gp) encoded by the *MDR-1* gene. Such a mechanism is easily verified in *in vitro* cultured cancer cells, but only scattered evidence has been provided for its actual occurrence in patients affected by solid tumors [1]. We previously demonstrated that ovarian carcinoma does not express the *MDR-1* gene and P-gp,

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based on a study of 50 primary untreated ovarian carcinomas [2]. Mozzetti et al. demonstrated scarcely detectable *MDR-1* gene expression, without finding any statistically significant differences between sensitive and resistant ovarian carcinoma patients who had been treated by platinum–paclitaxel-containing chemotherapy [3]. According to these results, it would seem that *MDR-1*/P-gp does not contribute to taxane resistance of ovarian carcinoma within the clinical setting.

Nearly a decade later, after identification of the paclitaxel-binding site in beta-tubulin, Giannakakou et al. [4] described point mutation in tubulin at the paclitaxel-binding site as being responsible for taxane resistance. A strong correlation between tubulin point mutation and resistance to taxane-containing therapy was demonstrated in lung cancer patients [5]. However, other studies have failed to confirm the presence of tubulin point mutations in resistant patients who have lung carcinoma or advanced ovarian carcinoma [3,6–8]. Therefore, the role of tubulin point mutations as a mechanism for taxane resistance remains uncertain.

An additional mechanism of resistance is the selective expression of beta-tubulin isotypes. In fact, beta-tubulin is encoded by a large multigene family, with the most significant differences at the carboxyl terminus region. Cleveland and Sullivan [9] used this region to devise a classification system to distinguish tubulin isotypes in vertebrates. In humans, at least six possible beta-tubulin isotypes have been identified and characterized for tissue expression [3]. Classes I and IVb are constitutively expressed in all tissues, whereas classes III, IVa and II are expressed typically in brain tissue, but only at low levels in other tissue, with a few exceptions. Class VI possesses the lowest degree of homology when compared with the others and is specifically expressed in the hematopoietic compartment. In this regard, overexpression of class III beta-tubulin mRNA and protein has been reported in paclitaxel-resistant cells including ovarian carcinoma [10–12]. However, discrepancies between *in vitro* and *in vivo* data have been reported [11]. Furthermore, there is no guarantee that mRNA levels of beta-tubulin expression are always correlated with protein levels [13]. Although overexpression of the class III beta-tubulin gene or protein within the clinical setting has been reported to be correlated with taxane resistance in ovarian carcinoma [3], lung cancer [14] and breast cancer [15], it is still important to elucidate precise detailed data for the protein levels of beta-tubulin isotypes in ovarian carcinoma using clinical samples.

In the present study, we evaluated protein levels of beta-tubulin isotypes (classes I, II, III and IV) in detail by immunohistochemical assay using clinical specimens of ovarian carcinoma, and we compared these data with the clinical features, including progression-free survival following taxane-based chemotherapy.

Materials and methods

Patients

Seventy-seven patients with primary ovarian carcinoma, who had undergone debulking surgery followed by chemotherapy at Kyushu University Hospital between 1994 and 2004, were examined. According to our selection criteria, we excluded the following patients: those patients (a) who were not treated by chemotherapy, (b) whose clinical information was not available, (c) with ovarian tumors which had a possibility of borderline malignancy, (d) with ovarian tumors which had a possibility of being metastatic tumors, (e) with ovarian tumors diagnosed as clear cell adenocarcinoma, (f) with concomitant extra-ovarian malignant tumors; patients were staged according to the International Federation of Obstetrics and Gynecology (FIGO) classification [16]. Fifty-four patients were subjected to chemotherapy using taxanes (52 patients treated by paclitaxel, 180 mg/m^2 body surface: 2 patients treated by docetaxel, 70 mg/m^2 body surface) and carboplatin (CBDCA). The dose of CBDCA was calculated by Calvert's formula. Twenty-three patients were treated by a taxane-free regimen which was composed of cisplatin (70 mg/m^2 body surface), epirubicin (50 mg/m^2 body surface) and cyclophosphamide (500 mg/m^2 body surface). Progression-free survival is defined as the interval from the date at initial surgery to the date at the diagnosis of progression. Primary tumors were classified according to recent WHO classification and graded as grade 1, 2 or 3 according to Silverberg's proposal, using extensively sampled paraffin-embedded samples [17]. We obtained written informed consent from all patients.

Antibodies

Monoclonal antibodies to beta-tubulin (class I, clone SAP.4G5, SIGMA-ALDRICH, SL; class II, clone JDR3B8, Biogenex, CA; class III, clone 5G8, Promega, WI; class IV, clone ONS1A, Biogenex, CA) were utilized, and the working dilution of each of the antibodies was 1:800, 1:400, 1:200 and 1:100, respectively. Skim milk-blended phosphate buffered saline (PBS) was utilized as the diluted solution for anti-class I, II and III beta-tubulin antibodies, whereas skim milk-free PBS was utilized for anti-class IV beta-tubulin antibody.

Immunohistochemistry

Surgically resected specimens prior to chemotherapy were fixed with 10% formalin and embedded in paraffin. Four-micrometer-thick sections on silane-coated slides were stained using the universal immunoperoxidase polymer method with a HISTOFINE kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. After deparaffinization, rehydration and inhibition of endogenous peroxidase, sections were exposed to the primary antibodies at 4 °C overnight. After incubation of the secondary antibody at room temperature, the sections were then finally incubated in 3 3′-diaminobenzidine, counterstained with hematoxylin, and mounted. For staining with anti-class I and III beta-tubulin antibodies, microwave heating was processed for the purpose of antigen retrieval. All the specimens for immunohistochemistry in this study were taken from primary ovarian tumors. Specimens taken from a metastatic site were not included.

Scoring of immunohistochemical results

We evaluated the proportion and intensity of the immunoreactive cells on the entire slides following the manner used by Allred et al. to evaluate the estrogen/progesterone receptors in breast cancer [18].

The proportional score (PS) is defined as follows:

0%=PS 0, 0<PS 1<1%, 1<PS 2<10%, 10%<PS 3<33%, 33<PS 4<67%, 67<PS 5<100%.

The intensity score (IS) is defined as follows:

IS 0=negative, IS 1=weak, IS 2=intermediate, IS 3=strong.

PS and IS were added to obtain a total score (TS) (range, 0, 2–8), and TS represents the immunohistochemical results. We believe that the heterogeneity of protein expression can be appropriately reflected in the immunohistochemical results by this scoring system.

Evaluation of the immunohistochemical results was scored by two pathologists (Y Oda and Y Ohishi) without knowledge of the clinical data of the patients.

Statistics

We used the statistical software StatView version 5.0 (SAS Institute Inc., Cary, NC). Associations between two dichotomous variables were evaluated by χ^2 statistics or Fisher's exact test. Differences in progression-free survival were analyzed using log-rank statistics. The influences of possible confounding factors for progression-free survival were analyzed by Cox proportional hazards regression model, using a non-stepwise method. Only p-values <0.05 were considered significant. p-values <0.1 were regarded as having propensity for association.

Results

Patients

The clinical and pathological characteristics of the 77 ovarian carcinomas were as follows.

The median age of the patients was 54.5 years (range, 36 to 77 years). Nine tumors were classified as stage I, 4 as stage II, 40 as stage III and 24 as stage IV. Stages I and II were regarded as early stage, whereas stages III and IV were regarded as advanced stage. Six tumors showed histological grade I, 46 grade II, and 25 grade III. Histologically, 66 tumors were serous adenocarcinoma, 4 were endometrioid adenocarcinoma, 3 were mixed malignant epithelial tumors, 3 were mucinous adenocarcinoma and 1 was adenocarcinoma, not otherwise specified. The maximum diameter of postoperative residual lesions in 20 patients was greater than 2 cm, whereas that in 52 patients was smaller than 2 cm. As for the remaining 5 patients, the medical records did not refer to the size of the residual lesions. The median progression-free survival was 621 days (range, 138–3717 days), whereas the median survival was 1012 days (range, 138–3717 days). The median follow-up period for those patients who are currently progression-free is 1347 days (range, 138–3717 days).

Immunohistochemical expression of beta-tubulin isotypes

Fig. 1 shows the results of immunohistochemical analysis of the representative tissues using anti-class I, II, III and IV beta-tubulin antibodies.

The immunohistochemical profiles of each of the beta-tubulin isotypes are summarized in the histogram (Fig. 2). As for the class II, III and IV beta-tubulin isotypes, the cut-off value of the immunohistochemical scores which divide high- or low-score categories was arbitrarily determined as follows, and the distribution is summarized in Table 1 in relation to the clinical factors.

class II beta-tubulin: scores 2–8=high level (present), score 0=low level (absent)
class III beta-tubulin: scores 5–8=high level, scores 0, 2–4=low level
class IV beta-tubulin: scores 7,8=high level, scores 0, 2–6=low level.

Class I beta-tubulin expression was not categorized since the expression levels of all the samples were consistently high (not less than score 7).

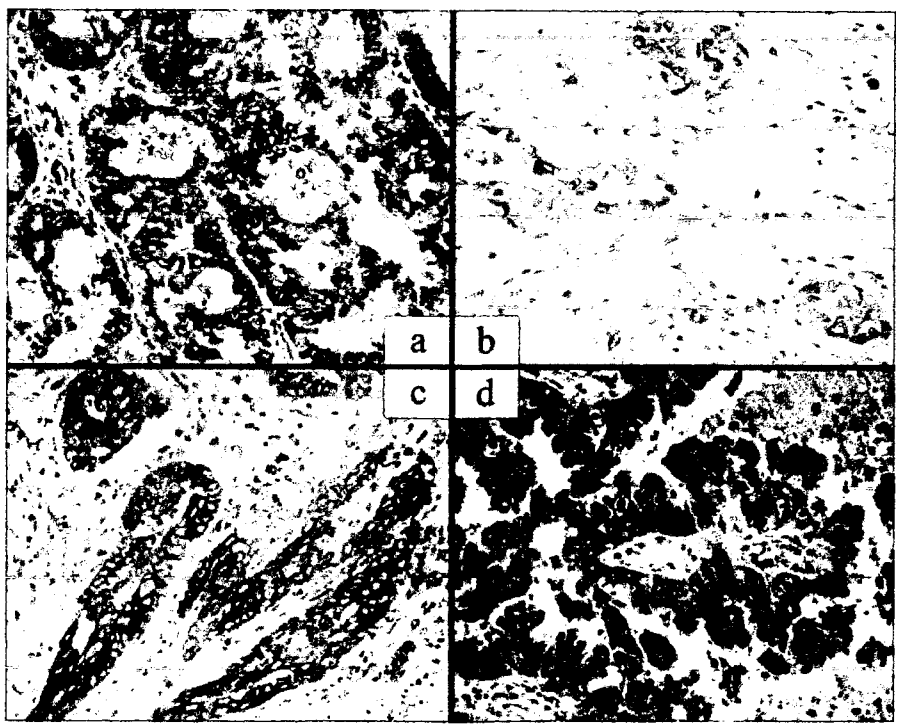

Fig. 1. Immunohistochemical results of the representative tissue sections. (a) Class I beta-tubulin. PS5+IS3=TS8, endometrioid type, stage Ic, PFS 1598 days. (b) Class II beta-tubulin. PS1+IS2=TS3, mixed type, stage Ia, PFS 903 days. (c) Class III beta-tubulin. PS3+IS3=TS6, serous type, stage IIIc, PFS 276 days. (d) Class IV beta-tubulin. PS5+IS3=TS8, serous type, stage IIIc, PFS 683 days. Each of the beta-tubulin isotypes was expressed in the cytoplasm of the cancer cells.

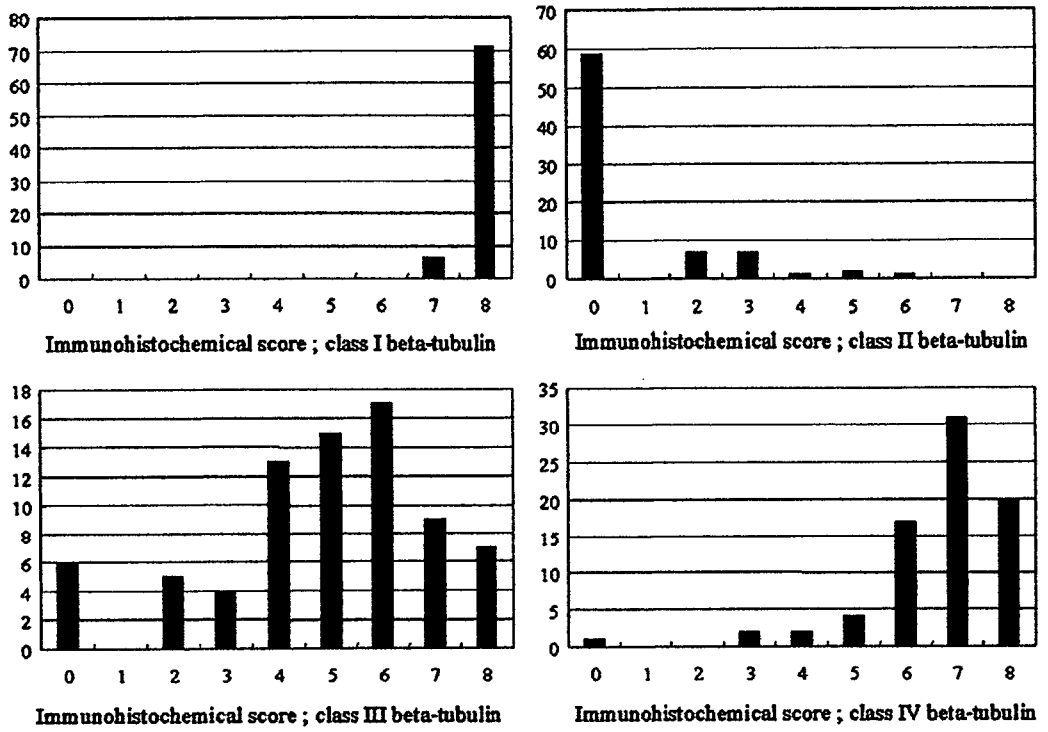

Fig. 2. Immunohistochemical profile of each of the beta-tubulin isotypes summarized in the histogram. The longitudinal axis represents the number of patients. The cut-off values dividing the high or low category of each of the isotypes were arbitrarily determined in reference to this histogram.

Comparison between beta-tubulin expression isotypes and clinical features

54 patients who received taxane-based regimen

The absence of class II beta-tubulin expression was significantly correlated with advanced stage ($p=0.024$), but high or low levels of the other isotypes demonstrated no significant correlations with clinical parameters, as summarized in Table 1. Serous phenotype was also significantly correlated with advanced stage ($p<0.0001$). The other data sets demonstrated no significant correlations or propensities.

Table 1
Clinicopathological characteristics of 77 ovarian carcinoma patients

	Taxane-based regimen ($n=54$)			Taxane-free regimen ($n=23$)		
	Class II High/ Low	Class III High/ Low	Class IV High/ Low	Class II High/ Low	Class III High/ Low	Class IV High/ Low
Age						
≥55	8/19	18/9	18/9	3/9	8/3	8/4
≤54	6/21	15/12	18/9	1/10	7/4	7/4
Stage						
Early	6/5 *	8/3	8/3	1/1	2/0	2/0
Advanced	8/35	25/18	28/15	3/18	13/7	13/8
Residual lesion						
≤2 cm	12/26	23/15	26/12	3/11	10/3	9/5
≥2 cm	1/10	6/5	8/3	1/8	5/4	6/3
Histology						
Serous	9/34	26/17	29/14	4/19	15/7	15/8
Others	5/6	7/4	7/4	0/0	0/0	0/0
Grade						
I	3/2	5/0	4/1	0/1	1/0	1/0
II, III	11/38	28/21	32/17	4/18	14/7	14/8

* Significant correlation ($p=0.024$).

By univariate analyses, the absence of class II beta-tubulin expression and advanced stage was significantly correlated with a short period of progression-free survival ($p=0.022$, 0.0031, respectively). Higher grade and serous phenotypes were prone to be associated with a short period of progression-free survival, although not significantly so ($p=0.065$, 0.055, respectively).

By multivariate analyses, only advanced stage was significantly correlated with a short period of progression-free survival ($p=0.019$; hazard ratio, 7.66; 95% confidence interval, 1.40–42.03). High level expression of class III beta-tubulin was prone to be associated with a short period of progression-free survival, although this finding was not statistically significant ($p=0.081$; hazard ratio, 2.17; 95% confidence interval, 0.91–5.20). The results of univariate and multivariate analyses for progression-free survival are summarized in Table 2.

23 patients with taxane-free regimen

There was no significant correlation or propensity between the absence of class II beta-tubulin expression and advanced stage. Power analysis demonstrated a power of 74%.

No other significant clinical correlations or propensities were demonstrated in any of the other data sets.

Discussion

The major obstacle to the successful medical treatment of cancer is the development of drug resistance to currently used

Table 2
Univariate and multivariate analyses for progression-free survival in 54 patients with ovarian carcinoma treated by taxane-based regimen

Confounding factors	Univariate (p)	Multivariate (p)	Hazard ratio	95% C.I.[a]
Age (≥55)	0.71	0.22	0.61	0.28–1.35
FIGO stage (advanced)	0.0031[b]	0.019[b]	7.66	1.40–42.03
Histologic grade (II/III)	0.065[c]	0.11	5.43	0.68–43.46
Residual lesion (>2 cm)	0.54	0.46	0.70	0.26–1.83
Histology (serous)	0.055[c]	0.73	0.78	0.19–3.16
Class II beta-tubulin (Low)	0.022[b]	0.47	1.49	0.50–4.45
Class III beta-tubulin (High)	0.69	0.081[c]	2.17	0.91–5.20
Class IV beta-tubulin (High)	0.16	0.61	0.80	0.34–1.88

[a] 95% confidence interval.
[b] Statistically significant.
[c] Having propensity to association.

chemotherapeutics. The mainstay of therapy for advanced ovarian cancer is provided by the taxane-based regimen.

The mechanisms of taxane resistance have previously been explained by *MDR-1*/P-gp overexpression, missense mutations of the tubulin gene and selective expression of beta-tubulin isotypes. Recent studies have demonstrated that the first 2 mechanisms are not likely to contribute to taxane resistance, and that selective class III beta-tubulin overexpression may be the most promising mechanism [3].

Numerous experimental results of *in vitro* data have shown a significant correlation between the overexpression of class III beta-tubulin gene/protein and taxane resistance [10–12], although a discrepancy between mRNA and protein levels of beta-tubulin expression has also been reported, thus indicating the presence of posttranscriptional regulation [13].

Although *in vitro* studies have contributed enormously to our understanding of this complex phenomenon, some have questioned the extent to which *in vitro* models can actually represent clinical phenomena. To obtain more clinically relevant results, human ovarian carcinoma xenografts or clinical samples have been evaluated, but the results have been conflicting [3,11].

The precise expression profile of each of the isotypes may be important to clarify the mechanisms of taxane resistance. However, to the best of our knowledge, protein levels of each of the isotypes of beta-tubulin have not been systematically assessed in ovarian carcinoma.

In the present study, we focused on the immunohistochemical expression of each of the isotypes of beta-tubulin protein using clinical samples from 77 ovarian carcinomas, and we evaluated the correlation with clinical features.

We demonstrated overall high levels of classes I and IV, intermediate levels of class III and very low levels of class II beta-tubulin protein expression in human ovarian carcinoma. This observation is similar to that described by Nicoletti et al. in that class I isotype was the predominant one in ovarian carcinoma cells [11]. However, their results demonstrated much lower levels of class II, III and IV expression, whereas we demonstrated relatively high levels of class IV beta-tubulin expression. This may be explained by differences in the materials and methods utilized: they estimated the mRNA levels of each of the isotypes using quantitative RT-PCR assay in cells or xenografts, whereas we analyzed protein levels in clinical human samples by immunohistochemistry.

In 54 ovarian carcinoma patients treated with taxanes, high expression of class III isotype was prone to be associated with a short period of progression-free survival in multivariate analyses ($p=0.081$). On the other hand, no such correlations or propensities were found in patients treated by the taxane-free regimen. These results suggest that the selective overexpression of class III isotype may play some part in the development of taxane resistance within the clinical setting since the progression-free period is believed to be one of the clinical measures of drug response.

Mozzetti et al. demonstrated that both mRNA and protein levels of class III isotype were markedly overexpressed in taxane-resistant ovarian carcinomas within the clinical setting [3], and their study may share certain similarities with ours in that possible association between class III beta-tubulin overexpression and taxane resistance was demonstrated using clinical specimens of ovarian carcinoma. Similar results have also been demonstrated with regard to lung cancer [14] and breast cancer [15] in clinical settings.

The exact mechanism by which an increased expression of class III beta-tubulin mediates drug resistance is still unknown. Lu and Luduena [19] have shown in cell-free assays that the removal of class III beta-tubulin through immunoprecipitation with specific antibodies enhances paclitaxel-induced microtubule polymerization. The physiologic role of this isotype seems to be that of enhancing the microtubule dynamic instability, thereby overcoming suppression of microtubule dynamicity by paclitaxel [20,21]. Hari et al. demonstrated that overexpression of the class III beta-tubulin gene confers paclitaxel resistance by reducing the polymerization rate of microtubules using mammalian cells [22].

However, we must interpret our results cautiously due to the limited sample size and the retrospective nature of our study. We should bear in mind that conflicting results do actually exist [11,23]. Ferrandina et al. demonstrated that class III beta-tubulin overexpression is significantly correlated with a short period of overall survival, but not with progression-free survival using clinical specimens of ovarian carcinoma from patients treated by taxane [23]. They speculated that class III beta-tubulin overexpression may contribute to a pure aggressive behavior rather than to a taxane-resistant phenotype. Conversely, we did not observe any correlations or propensities for association between class III beta-tubulin overexpression and overall survival (data not shown). Additional analyses are necessary to fully clarify the actual clinical significance of the overexpression of class III beta-tubulin protein. Furthermore, one of the chief points that needs to be discussed is whether a p value below 0.1 really represents the presence of a propensity for association.

We selected undoubted primary and malignant ovarian tumors since we were very concerned that contamination with borderline or metastatic tumors would significantly confuse our data. This is why our materials contained a relatively smaller number of endometrioid and mucinous phenotypes

which are sometimes difficult to confirm as to whether they are borderline or malignant tumors, or primary or metastatic tumors. Because the chemotherapy regimen of clear cell adenocarcinoma was different from that of our materials, we also excluded clear cell adenocarcinoma. However, a possibility that such decisions would create selection bias cannot be refuted.

We demonstrated that the progression-free survival of the class II beta-tubulin-absent group was significantly shorter than that of the class II beta-tubulin-present group by univariate analysis, and this result can be explained by the significant correlation with advanced stage which was an independent and strong predictor of a short period of progression-free survival.

Schnaeker et al. reported that matrix metalloproteinase (MMP)-2 and MMP-9 are localized almost perfectly on microtubules in melanoma cells [24]. This result suggests that microtubules contribute to the intracytoplasmic transport or exocytosis of MMPs which are zinc-dependent endopeptidases that cleave and degrade a wide spectrum of extracellular matrix components. The presence or absence of class II beta-tubulin may alter the function of microtubules and may affect the secretion of MMPs. This speculation may explain the significant correlation between the absence of class II beta-tubulin expression and advanced stage, although as yet this is simply a mere assumption.

In the early-stage group which consisted of 13 patients, 3 patients suffered a recurrence (unfavorable patients) while the remaining 10 patients are free of disease at present (favorable patients) (follow-up period: median, 1598 days; minimum, 562 days; maximum, 3717 days). Of the tumors taken from the 3 unfavorable patients, one tumor was class II beta-tubulin-present and the other two were class II beta-tubulin-absent. Class II beta-tubulin-absent tumors seem to have more aggressive tumor behavior in a very limited number of early-stage patients. However, progression-free and overall survival analyses demonstrated no clinical significance between the class II beta-tubulin-present group and the class II beta-tubulin-absent group in early-stage patients. Furthermore, class II beta-tubulin expression was not correlated with stage (stage I versus stage II). Further analysis is necessary in order to fully clarify the clinical significance of class II beta-tubulin expression.

To summarize our results, our findings suggest that selective overexpression of class III beta-tubulin seems to be associated with a short period of progression-free survival for ovarian carcinoma patients following taxane-based chemotherapy. This may support the hypothesis that selective overexpression of class III beta-tubulin contributes to taxane resistance within the clinical setting. To confirm our conclusion, further analysis is necessary within a systematically treated group comprising a larger number of patients.

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Alteration of dihydropyrimidine dehydrogenase expression by IFN-α affects the antiproliferative effects of 5-fluorouracil in human hepatocellular carcinoma cells

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Abstract

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the catabolism of 5-fluorouracil (5-FU) and its activity is closely associated with cellular sensitivity to 5-FU. This study examines the role of DPD in the antiproliferative effects of 5-FU combined with IFN-α on hepatocellular carcinoma (HCC) cells in culture and asks whether IFN-α could affect DPD expression. The combined action of IFN-α and 5-FU on three HCC lines was quantified by a combination index method. Coadministration of IFN-α and 5-FU showed synergistic effects against HAK-1A and KYN-2 but antagonistic effects against KYN-3. The cellular expression levels of DPD mRNA and protein were markedly up-regulated in KYN-3 cells by IFN-α but were down-regulated in HAK-1A and KYN-2. The expression of thymidylate synthase mRNA and protein was down-regulated by IFN-α in all three cell lines. Coadministration of a selective DPD inhibitor, 5-chloro-2,4-dihydroxypyridine (CDHP), enhanced the antiproliferative effect of 5-FU and IFN-α on KYN-3 ~4-fold. However, the synergistic effects of 5-FU and IFN-α on HAK-1A and KYN-2 were not affected by CDHP. The antiproliferative effect of 5-FU could thus be modulated by IFN-α, possibly through DPD expression, in HCC cells. Inhibition of DPD activity by CDHP may enhance the efficacy of IFN-α and 5-FU combination therapy in patients with HCC showing resistance to this therapy. [Mol Cancer Ther 2007;6(8):2310–8]

Introduction

5-Fluorouracil (5-FU) is widely used in the treatment of various gastrointestinal cancers and other types of tumor. It is converted to the active metabolite 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) and inhibits thymidylate synthase (TS) activity competitively through the formation of a ternary complex of FdUMP, TS, and 5,10-methylenetetrahydrofolate. Cancer cells with high levels of FdUMP and low levels of TS are thus known to be sensitive to 5-FU (1).

Dihydropyrimidine dehydrogenase (DPD) is a rate-limiting enzyme involved in the degradation of pyrimidine bases and pyrimidine-based antimetabolites, such as 5-FU, and so diminishes the antitumor activity of 5-FU. This catabolism occurs mainly in the liver. DPD activity shows wide variation in both cancer patients and the healthy population (2).

The human DPD gene (*DPYD*) is located on chromosome 1p22. It is a single copy 950-kb gene comprising 23 exons (3), in which 39 mutations and polymorphisms have been identified (4–6). Abnormalities of *DPYD* that decrease DPD activity are observed in 3% to 5% of the total population (7), and several patients with congenital DPD deficiency were reported as suffering from severe toxicity after the administration of 5-FU (8). DPD activity in tumor cells is critical to the antitumor effects of 5-FU (9), and its inhibition is expected to enhance these effects.

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. The most effective treatment for patients with HCC is the surgical resection of hepatic lesions. Local therapeutic approaches, such as transcatheter arterial embolization (10), percutaneous transhepatic ethanol injection (11), microwave coagulation (12), and radiofrequency ablation (13), are also effective. However, these therapies are not sufficient for patients with advanced HCC, for whom surgery is often not suitable and whose 5-year survival rate is extremely low (14). For patients with advanced HCC, the clinical response of

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almost every anticancer drug is insufficient, and several combination chemotherapies have been tried. Combined chemotherapy with 5-FU and IFN-α has been used previously for patients with advanced HCC, with improved therapeutic effects (15–17). However, these reports of positive effects are contradicted by a previously observed lack of antitumor activity accompanied by increased toxicity (18). To improve the therapeutic index of 5-FU and IFN-α combination therapy, it is therefore important to establish the cause of this occasional decrease in efficacy and increase in side effects.

Plausible mechanisms, such as an increase in FdUMP, TS inhibition rate, and thymidine phosphorylase activity, a decrease in TS levels, and altered 5-FU pharmacokinetics, have been suggested to explain the improved therapeutic effects of IFN-α and 5-FU (19–23). Takaoka et al. (24) showed that transcription of the *p53* gene is induced by IFN-α/IFN-β, accompanied by an increase in p53 protein level, and that the apoptotic response by IFN-β combined with 5-FU was enhanced. Milano et al. (25) reported that IFN-α inhibits DPD activity in human tumor cells, suggesting that inhibition of DPD activity could be involved in 5-FU–induced antiproliferative activity. Consensus IFN was shown to enhance the antiproliferative effect of 5-FU against hepatoma cells through down-regulation of DPD expression (23). By contrast, increased expression of DPD protein by IFN-γ was reportedly observed at a concentration equivalent to that in the sera of patients (26).

According to the presence or absence of synergism by the combination of 5-FU and IFN-α, we classified six human HCC cell lines into two groups: the S-group containing three cell lines, which showed a synergistic effect, and the A-group containing the remaining three cell lines, which showed additive effects (27). The expression levels of type I IFN receptor subunits were specifically up-regulated by 5-FU in all three cell lines of the S-group but not in those of the A-group (27). In this study, we asked whether DPD could limit the antiproliferative effect of 5-FU against HCC cells in culture when 5-FU was applied in combination with IFN-α. This work also shows that inhibiting DPD activity in HCC cells with high DPD levels improves the efficacy of 5-FU combined with IFN-α, following up-regulation by IFN-α.

Materials and Methods

Drugs

5-FU was purchased from Kyowa Hakko Kogyo Co. Ltd. (5-FU Injection 250 Kyowa) and natural human IFN-α was purchased from Otsuka Pharmaceutical Co. Ltd. (OIF). 5-Chloro-2,4-dihydroxypyridine (CDHP) was a gift from Taiho Pharmaceutical Co. Ltd.

Cell Lines

HCC cell lines, KYN-2, KYN-3, and HAK-1A (28, 29), were grown in DMEM (Nissui Seiyaku Co.) with 10% fetal bovine serum (FetalClone III, Hyclone) in a humidified atmosphere of 5% CO_2 at 37°C. We confirmed the expression of type I IFN receptor subunits 1 and 2 in these three HCC cell lines (27).

Cytotoxicity Tests

Cells were seeded into 96-well plates at 1,000 cells/100 μL/well and incubated overnight. On the following day, 100-μL aliquots containing IFN-α and/or 5-FU with or without CDHP were added to each well and cells were cultured for a further 5 days. The number of viable cells was estimated by assaying the activity of cellular succinate dehydrogenases using WST-8 reagent (Cell Counting Kit-8, Dojindo; ref. 30). We confirmed that untreated groups of KYN-2, KYN-3, and HAK-1A cells grew exponentially for 6 days under these experimental conditions (data not shown).

Combination Index Analysis

The combined effects of 5-FU and IFN-α were quantified using a combination index (CI) method developed by Chou and Talalay (31). This method involves plotting dose-effect curves, for each agent and their combination, using the median-effect equation: $fa / fu = (D / Dm)^m$, where D is the dose of the drug, Dm is the dose required for a 50% effect (equivalent to IC_{50}), fa and fu are the affected and unaffected fractions, respectively ($fa = 1 - fu$), and m is the exponent signifying the sigmoidicity of the dose-effect curve.

In this study, relative concentrations (RC) of IFN-α and 5-FU, determined as (concentration) / (IC_{50} value), were used for analysis. The computer software Xlfit version 2.0.6 (ID Business Solutions Ltd.) was used to calculate the values of Dm and m. The CI used for the analysis of the drug combinations was determined by the isobologram equation for mutually nonexclusive drugs that have different modes of action: $CI = (D)_1 / (Dx)_1 + (D)_2 / (Dx)_2 + (D)_1(D)_2 / (Dx)_1(Dx)_2$, where $(D)_1$ and $(D)_2$ are RCs of drugs 1 and 2 and x is the percentage of inhibition. Combination indices $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additive effects, and antagonism, respectively.

Quantitative Real-time Reverse Transcription-PCR

Total RNA was extracted using Isogen (Nippon Gene Co., Ltd.) and reverse transcribed using a reverse-transcription system (Promega Corp.) according to the manufacturer's instructions. Quantitative real-time reverse transcription-PCR was done with an ABI Prism 7300 (PE Applied Biosystems). The primers used were as follows: TS 5′-GAATCACATCGAGCCACTGAAA-3′ (forward primer), 5′-CAGCCCAACCCCTAAAGACTGA-3′ (reverse primer), and 5′-(FAM)TTCAGCTTCAGCGAGAACCCAGA(TAMRA)-3′ (probe) and DPD 5′-AATGATTCGAAGAGCTTTTGAAGC-3′ (forward primer), 5′-GTTCCCCGGATGATTCTGG-3′ (reverse primer), and 5′-(FAM)TGCCCTCACCAAAACTTTCTCTCTTGATAAGGA(TAMRA)-3′ (probe). Primers and Taqman probes for glyceraldehyde-3-phosphate dehydrogenase were prepared by Assay-on-Demand Gene Expression Products (PE Applied Biosystems).

Western Blotting

HCC cells were cultured for 48 h with 0, 20, 100, or 500 IU/mL IFN-α. Total protein was extracted using a protein extraction reagent (M-PER, Pierce) supplemented with protease inhibitors (Halt Protease Inhibitor

Cocktail kit, Pierce). Cell lysates were loaded into 7.5% SDS-polyacrylamide gels. After electrophoresis, the separated proteins were electrotransblotted onto polyvinylidene difluoride membranes (Immobilon-P membrane, Millipore). After blocking, membranes were probed with antihuman DPD polyclonal rabbit antibody and antihuman TS monoclonal mouse antibody (gifts from Taiho Pharmaceutical). The proteins were visualized using horseradish peroxidase–conjugated antibodies (Pierce) followed by enhanced chemiluminescence (Pierce). The intensity of luminescence was quantified using an image analysis system (LAS-1000, Fuji Film).

Enzyme Assay for DPD Activity

DPD activity was measured using [6-^{14}C]5-FU as a substrate (32, 33). Cells were homogenized and centrifuged at 105,000 × *g* for 60 min at 4°C, and the supernatant was used for assay. A reaction mixture containing 10 mmol/L potassium phosphate (pH 8.0), 0.5 mmol/L EDTA, 0.5 mmol/L 2-mercaptoethanol, 2 mmol/L DTT, 5 mmol/L $MgCl_2$, 20 μmol/L [6-^{14}C]5-FU, 0.1 mmol/L NADPH, and 25 μL of cell extract in a total volume of 50 μL was incubated at 37°C for 30 min. After chemical hydrolyzation and neutralization using KOH and $HClO_4$, a 5-μL aliquot was applied to a TLC plate (2.5 × 20 cm, silica gel 60 F_{254} plate, Merck) and developed with a mixture of ethanol and 1 mol/L ammonium acetate (5:1, v/v) and diethylether, acetone, chloroform, and water (50:50:40:1, v/v). DPD activity was determined as the sum of the products converted from 5-FU (i.e., dihydrouracil, 2-fluoro-β-ureidopropionic acid, and 2-fluoro-β-alanine) that were visualized and quantified with an imaging analyzer (BAS-2000, Fujix).

Results

Comparison of Drug Sensitivity and mRNA Levels of DPD and TS in Three HCC Cell Lines

The sensitivities of three HCC lines, HAK-1A, KYN-2, and KYN-3, to separately administered 5-FU and IFN-α were determined as IC_{50} values. KYN-3 was the most resistant to 5-FU of the three HCC lines. The IC_{50} values of IFN-α in HAK-1A, KYN-2, and KYN-3 cells were 720, 510, and 24 IU/mL, respectively. KYN-3 cellular sensitivity to IFN-α was therefore approximately 30-fold and 20-fold higher than in HAK-1A and KYN-2 cell lines, respectively. The IC_{50} value of IFN-α in HepG2 cells established from human hepatoblastoma and widely used in experiments was found to be over 10,000 IU/mL (data not shown).

Sensitivity was then compared with mRNA levels of DPD and TS, and DPD activity (as conversion rate from [6-^{14}C]5-FU to its metabolites) in the three cell lines, which were shown to be broadly comparable (Table 1). DPD mRNA levels relative to those of KYN-3 cells, taken as 100%, and those of TS relative to HAK-1A, taken as 100%, are shown in Table 1. The cellular level of TS mRNA in KYN-2 cells was much lower than in the other two lines. Basal DPD activity in KYN-3 cells was approximately 5.5-fold and 9.1-fold higher than in HAK-1A and KYN-2 cell lines, respectively.

Quantitative Analysis of the Combination Effects of 5-FU and IFN-α

Dose-response curves of 5-FU alone and in combination with various concentrations of IFN-α are shown in Fig. 1A to C. Because sensitivities to IFN-α and 5-FU alone were different for each HCC cell line, RCs to the IC_{50} value were used. As the concentration of combined IFN-α was increased, the dose-response curves of 5-FU were shifted down in an IFN-α concentration-dependent manner in all three HCC cell lines. For instance, following cotreatment with 312 IU/mL (RC = 0.45) IFN-α,the dose-response curve of HAK-1A to 5-FU was significantly shifted down and the 5-FU IC_{50} value of 2.3 μmol/L was reduced to 0.28 μmol/L. However, there were clear differences between the three HCC cell lines. For KYN-3 cells, after treatment with the equivalent RC, 0.42 (8.0 IU/mL) IFN-α,the dose-response curve of 5-FU was significantly shifted down, but the 5-FU IC_{50} value of 9.6 μmol/L was only reduced to 5.5 μmol/L.

An enhancement factor (EF) was defined to evaluate synergism between 5-FU and IFN-α, based on a 50% antiproliferative effect, as EF = 1 / [(RC of IFN-α) + (RC of 5-FU)]. When EF is 1, this combined effect is additive; values >1 or <1 imply synergistic or antagonistic effects, respectively. EF values of HAK-1A and KYN-2 were >1 at almost all combined doses, but EF values of KYN-3 at all tested combined doses were closer to 1. The combined effect of 5-FU and IFN-α on HAK-1A and KYN-2 cells was thus judged to be synergistic and that on KYN-3 to be additive, consistent with our previous study (27).

Table 1. HCC cell sensitivities to 5-FU and IFN-α, DPD, and TS mRNA expression levels and DPD activities

Cell line	IC_{50}*		Relative mRNA levels†		DPD activity‡ (pmol/min/mg protein)
	5-FU (μmol/L)	IFN-α (IU/mL)	DPD	TS	
HAK-1A	2.1	720	15	100	2.8 ± 0.9
KYN-2	1.8	510	23	14	1.7 ± 0.7
KYN-3	9.8	24	100	46	15.5 ± 1.6

*The IC_{50} value that caused 50% growth inhibition was calculated from the log-logit regression line. The assays were carried out in quadruplicate. Experiments were repeated twice with essentially similar results.

†DPD mRNA levels are shown relative to those of KYN-3 cells, taken as 100%, and TS mRNA levels are shown relative to those of HAK-1A cells, taken as 100%.

‡Determinations were carried out in triplicate and data represent mean ± SD.

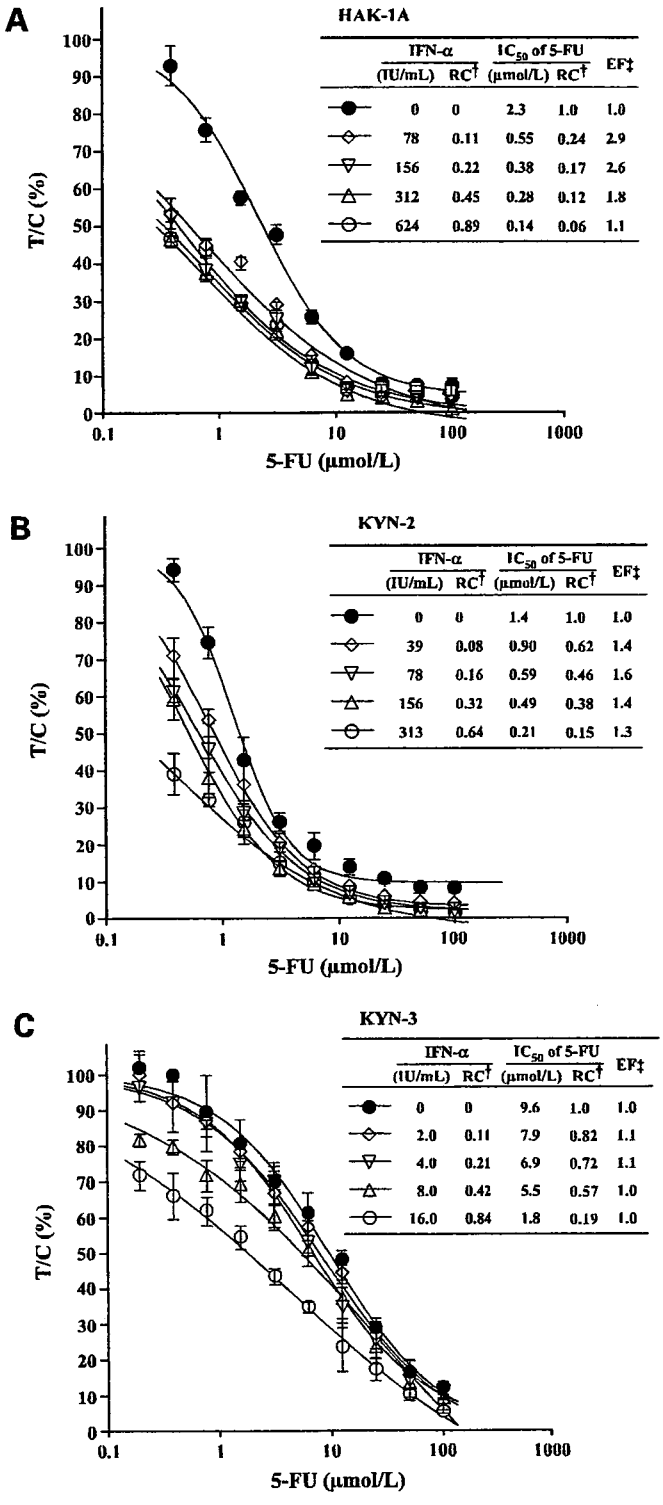

Figure 1. Antiproliferative effects of 5-FU and IFN-α on HAK-1A (**A**), KYN-2 (**B**), and KYN-3 (**C**) cells. Assays were carried out independently in quadruplicate. *Points,* mean; *bars,* SD. †, RC = (concentration) / (IC_{50} value). In this experiment, IC_{50} value of IFN-α against HAK-1A, KYN-2, and KYN-3 was 700, 490, and 20 IU/mL, respectively. ‡, EF = 1 / [(RC of IFN-α) + (RC of 5-FU)]. EF > 1, synergistic; EF = 1, additive; EF < 1, antagonistic. Experiments were repeated twice with similar results.

The synergism of IFN-α and 5-FU at various fractional efficacies was then quantified using the CI methods of Chou and Talalay (31). The antiproliferative effects of 5-FU on HAK-1A and KYN-2 cells were synergistically enhanced by IFN-α, indicated by CI values of <1 at most fractional effects (Fig. 2A and B). By contrast, the CI value in KYN-3 cells was >1 for fractional effects of 0.7 and under (Fig. 2C). The combined effect of IFN-α and 5-FU against KYN-3 thus seems to be antagonistic, except at very high fractional effect levels. These data show that the degree of synergism between IFN-α and 5-FU against KYN-3 cells (with a higher CI value) is weaker than against HAK-1A and KYN-2 cells over a large range of fractional effects.

Altered Expression of mRNA and Protein of TS and DPD by IFN-α

Cellular mRNA levels of DPD and TS were examined following treatment with or without 500 IU/mL IFN-α for 24 h (Fig. 3A and B). IFN-α treatment resulted in an ~7.5-fold increase in DPD mRNA levels in KYN-3 cells while reducing DPD mRNA levels in HAK-1A and KYN-2 cells to approximately one quarter and one third of that in IFN-α untreated cells, respectively (Fig. 3A). TS mRNA levels in all three HCC cell lines decreased after treatment with IFN-α to ~60% of those in untreated cells (Fig. 3B).

Cellular protein levels of DPD and TS were also determined by Western blot analysis. The intensities of blotted bands were normalized by that of β-actin, and fold increase was measured as the relative intensity to that of untreated cells, taken as 1.0. Protein extracts of 10 μg/lane were loaded for KYN-3 cells, and 50 μg/lane were loaded for both HAK-1A and KYN-2 cells (Fig. 4). DPD protein levels were roughly comparable with mRNA levels and DPD activity in all three cell lines. Expression of DPD protein, molecular weight of 110,000, was down-regulated in HAK-1A and KYN-2 cells when treated with over 100 IU/mL IFN-α for 48 h. Conversely, that in KYN-3 cells was up-regulated after treatment with over 20 IU/mL IFN-α for 48 h in a concentration-dependent manner (Fig. 4). Expression of TS protein, molecular weight of 36,000, was down-regulated to a similar extent in a concentration-dependent manner in all three HCC cell lines when treated with IFN-α.

Effect of a DPD Selective Inhibitor on the Antiproliferative Effect of 5-FU and IFN-α

The combined antiproliferative effects of 5-FU and IFN-α can be modulated by treatment with a DPD competitive inhibitor, CDHP. We first examined the effect of CDHP on DPD activity in three HCC cell lines. KYN-3 cell extracts showed relatively high DPD activity of ~15 pmol/min/mg protein (consistent with Table 1 findings). By contrast, DPD activity in HAK-1A and KYN-2 cells was very low with a marginal limit of evaluation for enzyme activity. DPD activity in KYN-3 cells was reduced to 40%, 7%, and 5% of basal activity after treatment with 0.7, 7.0, and 70 μmol/L CDHP, respectively (Fig. 5). There seemed to be almost no marked inhibition of DPD activity by CDHP in the other two cell lines, possibly due to their low enzyme activities.