治療方法

1●TAE前処置

TAEに向け，治療目的や内容について医師などから説明されます．クリニカルパスが作成されている場合，それを用い治療前後の予定された経過を説明します．看護師は患者さんのTAEに対する理解度の確認や疑問の有無について確認し，安心してTAEが受けられるように援助します．前日の処置には術野の除毛，シャワーまたは入浴，尿量測定の開始，足背動脈のマーキング，IDバンドの着用があります．また同意書の有無を確認します．

2●TAE当日

TAE開始までは病棟内フリーとし，朝から絶食，血管造影開始3時間前より絶飲とします．出棟前に血管確保を行い，バイタルサインを測定，必要に応じて前投薬を投与します．

3●TAEの実際

大腿動脈周囲に局所麻酔を行い，大腿動脈を穿刺，イントロデューサーを留置します．そのイントロデューサーを通じてカテーテルを挿入し，血管造影を行い，肝動脈の解剖，腫瘍の状態（部位，個数），栄養血管の同定を行います．また，経上腸間膜動脈性門脈造影を行い，門脈本幹閉塞や肝内門脈枝内の腫瘍栓の有無を調べます．前述したように側副血行路のない門脈本幹閉塞例ではTAEは禁忌です．

塞栓予定の肝動脈までカテーテルを進め，塞栓術を行います（図）．通常，抗がん剤とリピオドールを混和したものとゼラチンスポンジなどの塞栓物質を用いて塞栓します．TAEは心電図や血圧のモニター下に行われます．造影剤，血管拡張剤，抗がん剤や塞栓物質注入時に後述のような合併症が起こることがあるので，バイタルサインや全身状態を注意深く観察します．

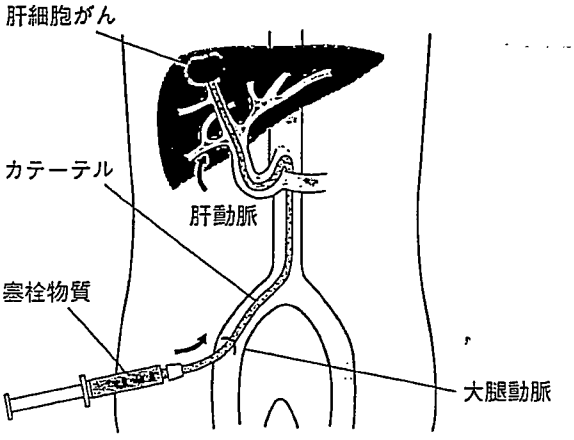

図　肝動脈塞栓療法

4●TAE後

カテーテル抜去後，止血のために10～30分間，穿刺部を圧迫します．止血確認後，伸縮絆創膏などを用いて圧迫止血します．帰室後，数時間から12時間，穿刺側の下肢を屈曲しないようにしてベッド上で安静を保ちます．後述の合併症の観察，バイタルサイン測定，穿刺部の出血の有無，足背動脈の触診，尿量測定を行います．またベッド上安静中は，必要時排泄や食事の介助，体位変換，腰痛があれば疼痛緩和などを行います．

治療中，治療後に気を付けるべき合併症

- 血圧上昇，血圧低下
- 腹痛，嘔気，嘔吐，発熱
- 穿刺部出血，血栓形成
- 肝機能障害
- 肝梗塞，胆管炎，胆嚢炎，肝膿瘍，肝がん破裂

1●血圧上昇，血圧低下

塞栓物質注入時，しばしば腹痛が発生し，血圧上昇をきたす場合があります．血管造影下CTや経上腸間膜動脈性門脈造影で使用する血管拡張剤による副作用や造影剤による副作用で一過性の血圧低下をきたすことがあるので，患者さんの状態把握が重要です．

2●腹痛，嘔気，嘔吐，発熱

TAE後には，一過性の腹痛，嘔気，嘔吐，発熱が生じます．これらは通常数日以内に改善することが多いですが，鎮痛処置や制吐薬の使用（抗がん剤使用の場合）も考慮します．

3●穿刺部出血，血栓形成

穿刺部の圧迫が不適切であったり，血小板数の低い患者さんでは穿刺部の出血がみられることがあるので注意を要します．また，穿刺部の血腫形成や穿刺動脈の血栓形成がみられると足背動脈の触知が困難となるので，定期的に観察し異常の早期発見に努めなければなりません．

4●肝機能障害

広範囲のTAEの場合，肝機能障害が惹起される場合があります．

5●肝梗塞，胆管炎，胆嚢炎，肝膿瘍，肝がん破裂

塞栓範囲の門脈血流が途絶あるいは減少している場合，肝梗塞が起こることがあります．胆管や胆嚢への動脈血流の減少，途絶の結果，胆管炎や胆嚢炎，さらに肝膿瘍がひき起こされる場合があるので，TAE後しばらくの間は注意を要します．まれに，肝がん破裂をきたす場合があり，この際には急激に発症する腹痛や貧血がみられ，ショック状態に陥ることがあります．

観察項目checkリスト●

- 血圧，脈拍，体温，尿量
- 穿刺部の出血
- 足背動脈の拍動触知
- 腹痛，嘔気，嘔吐の有無

文献

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Original Article

New chemotherapy for patients with advanced hepatocellular carcinoma: Pilot study of β-interferon and doxorubicin one-shot intra-arterial chemotherapy

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Background: Patients with advanced hepatocellular carcinoma (HCC) need an effective treatment modality because of the poor prognosis of the disease. From an *in vitro* study, β-interferon (IFN-β) has been reported to enhance the antiproliferative effects of doxorubicin on HCC cell lines. In the present study, we investigated the therapeutic effects of combined IFN-β and doxorubicin intra-arterial injection therapy on patients with advanced HCC.

Methods: IFN-β (3 MIU) and doxorubicin (10 mg/bodyweight) were given by one-shot intra-arterial injection through an arterial port to patients with advanced HCC. One treatment course consisted of three intra-arterial injections per week for 4 weeks. Three courses were conducted and evaluation was done monthly.

Results: Eleven patients with advanced HCC were treated with combined IFN-β and doxorubicin. One patient entered complete remission (CR), seven patients were evaluated as having stable disease (SD) and three as having progressive disease (PD). The mean overall survival was 10 months. The mean survival for CR and SD patients was 15 months, and that for PD patients was 6 months ($P = 0.0464$, log–rank test). Decrease of serum total bilirubin was observed for all patients.

Conclusion: Combined IFN-β and doxorubicin intra-arterial therapy offers an effective chemotherapy option for patients with advanced HCC by improving liver function and having tolerable side-effects.

Key words: advanced hepatocellular carcinoma, β-interferon, doxorubicin, intra-arterial injection

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is principally associated with hepatitis B virus (HBV) or hepatitis C virus (HCV), and its incidence is especially high in Asia and Africa.[1] Recently, its incidence has been increasing in Europe and America.[2,3] There are various options for treatment of HCC, including radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), transcatheter arterial embolization (TAE) using interventional radiology (IVR), surgical resection, and liver transplantation.[4] However, the prognosis is poor for patients with advanced hepatic carcinomas, which develop in multiple segments in the liver and/or are accompanied by portal vein tumor thrombus, because no efficacious treatment modality has yet been developed.[5] Recently, for patients with advanced HCC without metastatic foci whose performance status (PS) is good, approximately 50% effectiveness has been reported for combined α-interferon (IFN-α) and 5-fluoruracil (5-FU) arterial injection therapy.[6,7] For patients with poor liver function who cannot accept IFN-α and 5-FU combination therapy, a new chemotherapy regimen is needed. Thus, we designed a protocol that minimizes hepatic toxicity and also enables one-shot arterial injection for patients with advanced HCC, who are not candidates for operation, liver

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transplantation, or local treatment such as IVR, PEIT or RFA due to the number of tumors, portal vein thrombosis, or liver dysfunction (BCLC staging system B or C).[8]

β-interferon (IFN-β) is usually given by injection into the bloodstream and has fewer side-effects than IFN-α.[9] Recently, an *in vitro* study has shown that IFN-β could suppress the proliferation of HCC more strongly than IFN-α both alone and in combination with anticancer agents.[10] In particular, the antitumor agent doxorubicin showed synergism with IFN-β in the antiproliferation effect against HCC using HCC cell lines.[11] As myocardial damage and hepatic toxicity are the main side-effects of doxorubicin,[12,13] a small-volume one-shot arterial injection was selected for giving IFN-β. This led us to design a new chemotherapy regimen of combined IFN-β and doxorubicin intra-arterial injection therapy. The present study was conducted to determine whether this combined chemotherapy could be used for outpatient treatment after a short hospital stay in order to maintain the patient's quality of life (QOL) with fewer side-effects.

METHODS

Patient enrollment

PATIENTS WITH CIRRHOSIS and advanced HCC who were enrolled in this study were not eligible for surgical resection, liver transplantation or local treatment such as IVR, PEI or RFA because of diffuse or multiple tumors in both lobes with or without portal vein tumor thrombus and/or impaired liver function due to cirrhosis. To realize chemotherapy on an outpatient basis, patients with PS 0 or 1 were selected. Informed consent was obtained after explaining the purpose of the study and possible side-effects. Clinical tumor stages of patients with HCC were evaluated by abdominal contrast enhanced computed tomographic (CT) scans, magnetic resonance images (MRI) or angiography. Other criteria were a neutrophil count $\geq 1000/mm^3$, platelet count $\geq 40\,000/mm^3$, serum level of creatinine ≤1.4 mg/dL, total bilirubin of ≤3.5 mg/dL, and no abnormalities of cardiac function by ultrasound and electrocardiography. The exclusion criteria included intractable pleural effusion or ascites, severe infectious disease, severe myocardial damage, severe impairment of intelligence, encephalosis, metastasis to the central nervous system, hemorrhage from varicose veins within 1 month prior to enrollment, and pregnancy.

Therapeutic design

All of the enrolled patients had a catheter placed by gastroduodenal artery (GDA) coil or other method and a port implanted subcutaneously. One course of chemotherapy consisted of one-shot intra-arterial injection of IFN-β (3 MIU) and doxorubicin (10 mg/bodyweight) through the port, three times per week for 4 weeks. Three courses were conducted, when possible, and monthly evaluation of chemotherapy effects on HCC was based on serum tumor markers and CT scans.

Evaluation of therapeutic effects

The antitumor effect was evaluated by tumor volumes using contrast enhanced CT scans every 4 weeks from the start of combined IFN-β and doxorubicin intra-arterial injection therapy. The antitumor effect and toxicity were evaluated according to National Cancer Institute Common Toxicity Criteria (NCI-CTC)[14] and Response Evaluation Criteria in Solid Tumors (RECIST)[15] guidelines. Peripheral blood cells, biochemical tests, serum levels of α-fetoprotein (AFP) and/or PIVKA-II were examined every 4 weeks. The overall survival was calculated from the first treatment until death or the final day of follow up. The primary end-point of the current study was the development of toxicity and overall survival.

The criteria of complete response (CR), stable disease (SD) and progressive disease (PD) were as follows: CR, complete disappearance of tumors and no evidence of new lesions; SD, < 50% reduction or < 25% increase of tumor volume and no evidence of new lesions; PD, ≥25% increase of tumor volume, evidence of new lesions, or rise in tumor markers.

Statistics

The overall survival time from the start of the chemotherapy was analyzed by the Kaplan–Meier method and differences in survival were evaluated by log-rank tests.

RESULTS

Patient characteristics

ELEVEN PATIENTS WERE enrolled at Osaka University Hospital between November 2003 and August 2005. HCC was diagnosed by contrast-enhanced CT scan or MRI. Angiography and pathological diagnosis were not done. The serum levels of AFP and PIVKA-II were elevated. The pretreatment characteristics of enrolled patients are shown in Table 1.

Table 1 Pretreatment characteristics of patients with advanced hepatocellular carcinoma

No.	Age (years)	Sex	Etiology	Child–Pugh grade	Portal venous thrombosis (Vp)	Previous treatment
1	56	M	HBV/HCV	B	+	TAE
2	78	M	HCV	A	+	TAE, RFA
3	73	M	HBV	A	–	Operation, TAE
4	58	M	HCV	B	–	TAE
5	71	M	HCV	B	–	TAE
6	49	M	HCV	C	+	TAE, RFA
7	69	M	Non B/non C	B	+	None
8	63	M	HBV	A	+	TAE, RFA
9	62	F	HCV	B	–	TAE
10	61	M	HCV	A	+	TAE, RFA
11	56	M	HCV	A	–	None

HBV, hepatitis B virus; HCV, hepatitis C virus; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.

All patients were enrolled after being diagnosed as having liver cirrhosis by biochemical tests and/or radiological findings. Histological confirmation of liver cirrhosis was not done. The liver function of patients with cirrhosis was classified according to Child–Pugh grading criteria. Pretreatment tumor stages of patients with advanced HCC were classified according to the American Joint Committee on Cancer (AJCC) Tumor-Lymph Node Metastasis (TNM) classification system,[16] and according to the Cancer of the Liver Italian Program (CLIP) score[17] (Table 2). Seven patients had HCV infection, two had HBV, one had both HBV and HCV. One patient suffered from cirrhosis with neither HBV nor HCV infection.

Tolerability and side-effects

Eleven patients were started with intra-arterial administration of 3 MIU IFN-β and 10 mg doxorubicin. The median period of combined chemotherapy was 11 weeks (range 8–12 weeks). The dose of doxorubicin was reduced from 10 mg/bodyweight to 5 mg/bodyweight for two patients (nos. 2 and 6) because of grade 3 and 4 neutropenia. A 78-year-old man (no. 2) developed grade 4 neutropenia after the first course, and doxorubicin was reduced to 5 mg/bodyweight and granulocyte-colony stimulating factor (G-CSF) was given, and then grade 4 stomatitis appeared after two courses leading to discontinuation of the chemo-

Table 2 Therapeutic effect according to RECIST on patients and tumor stages of HCC patients according to the CLIP score and TNM classification system

No.	T-Bil (mg/mL)	AFP (ng/mL)	PIVKA II (mAU/mL)	CLIP score	TNM	Duration of therapy	Therapeutic effect	Prognosis
1	1.9	<5.3	<40	4	III	3 cycles	SD	15 M Dead
2	1.7	2 145	<40	4	IVA	2 cycles	PD	6 M Dead
3	0.6	24	148	1	III	3 cycles	SD	8 M Dead
4	3.3	24	140	2	III	3 cycles	SD	35 M Alive
5	1.6	25	462	3	III	2 cycles	SD	6 M Dead
6	2.1	10 400	32 852	6	IVB	3 cycles	SD	6 M Dead
7	1.3	226 820	12 317	5	IVA	3 cycles	PD	5 M Dead
8	2.4	582	63	3	IVA	3 cycles	CR	20 M Alive
9	2.9	41	1 397	2	IVA	2 cycles	SD	12 M Dead
10	0.7	255	1 341	3	III	3 cycles	PD	10 M Dead
11	2.4	309	13 900	1	III	3 cycles	SD	25 M Alive

AFP, α-fetoprotein; CLIP score, Cancer of the Liver Italian Program score; CR, complete remission; PD, progressive disease; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease; T-Bil, total bilirubin; TNM, Tumor-lymph Node Metastasis classification system.

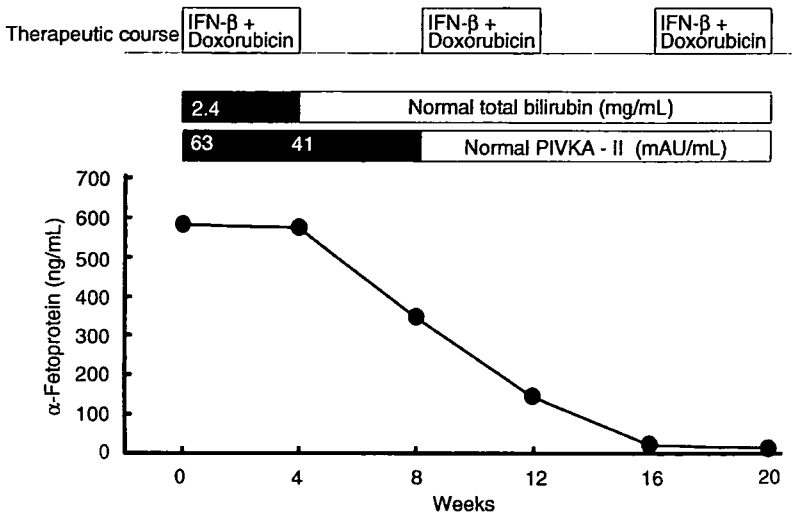

Figure 1 Time course of tumor markers in a complete remission case. A 63-year-old man with diffused type advanced hepatocellular carcinoma (HCC) (no. 8) was treated with three courses of combined β-interferon (IFN-β) and doxorubicin intra-arterial injection therapy without severe side-effects. Serum levels of PIVKA-II decreased after the first course of combined chemotherapy, entered the normal range during the second course and remained in the normal range after three courses. The serum level of α-fetoprotein decreased after the second course and entered the normal range 1 month after three courses of combined therapy. No HCC lesions were detected in the patient's liver by contrast enhanced CT scans and MRI after three courses of combined chemotherapy and 6 months later.

therapy. A 71-year-old man (no. 5) and a 62-year-old woman (no. 9) with Child–Pugh grade B complained of severe fatigue after two courses, and the chemotherapy was stopped. They had been treated by TAE for the tumors more than five times previously. Previous treatments, especially transarterial chemoembolization (TACE) may have affected the severity of the toxicity of the present combined chemotherapy regimen, although other factors such as age and Child–Pugh grade can be considered as having affected the development of intolerable side-effects. Discontinuation of drug therapy led to quick recovery from the adverse reactions. Of the eight remaining patients, three dropped out of the study and five completed three courses of treatment.

Therapeutic effects of combined intra-arterial IFN-β and doxorubicin injection therapy

All patients had advanced HCC, seven with and four without portal thrombus. All HCC were evaluated for volume changes by contrast-enhanced CT scans after 8 or 12 weeks. A 63-year-old man (no. 8) with HBV infection showed significant reduction of AFP and PIVKA-II into the normal range. Diffuse HCC disappeared after three courses of combined IFN-β and doxorubicin intra-arterial injection therapy, being confirmed by contrast-enhanced CT scan and MRI. Thus, we concluded that patient no. 8 had attained CR (Fig. 1).

All patients showed a high serum level of AFP and/or PIVKA-II before treatment (Table 2). The serum levels of AFP and/or PIVKA-II decreased after one course of combined chemotherapy in all patients. However, the CT scans demonstrated no significant volume reduction of HCC in seven patients, and tumor enlargement in three. Seven patients were classified as SD and three as PD from contrast-enhanced CT scans (Table 1).

Overall survival

All of the patients were observed from November 2003 to October 2006. The estimated duration of overall median survival was 10 months (Fig. 2a). The mean survival time was 15 months for CR and SD patients, which is significantly longer than 6 months for PD patients ($P = 0.0464$, log-rank test) (Fig. 2b). The mean survival time of only SD patients (12 months) was not significantly longer than that for PD patients ($P = 0.0786$, log-rank test). The one-year survival rate for CR and SD patients was 62.5% (5/8) and that for PD was 0% (0/3). The progression-free survival time for CR or SD was longer than that for PD ($P = 0.0004$, log-rank test)

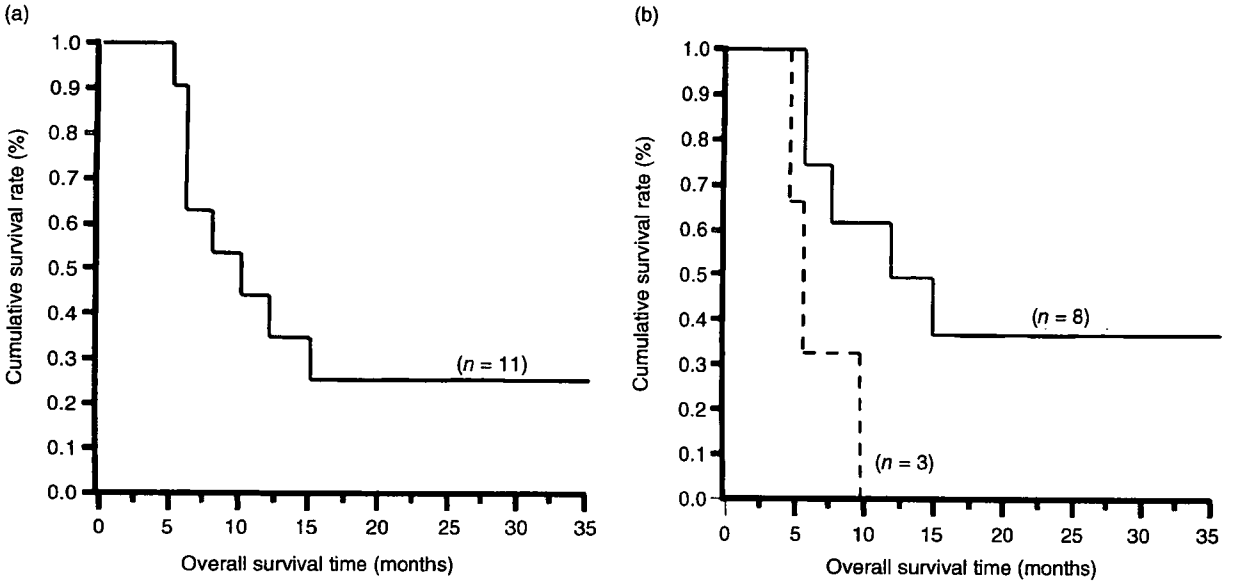

Figure 2 Overall survival periods of patients with advanced hepatocellular carcinoma who received combined β-interferon (IFN-β) and doxorubicin intra-arterial injection therapy. (a) Overall survival periods of 11 patients who received combined IFN-β and doxorubicin intra-arterial injection therapy. The mean survival period was 10 months. (b) Overall survival periods of seven patients with stable disease (SD) and three with progressive disease (PD) after combined IFN-β and doxorubicin intra-arterial injection therapy. The mean survival period was 15 months for SD patients and 6 months for PD patients. (——), CR-SD; (- - -), PD. CR, complete response. ($P = 0.0464$, log-rank test).

(Fig. 3). Eight patients died of liver failure, including five SD and three PD patients. A 73-year-old man (no. 3) died of sepsis that developed from catheter problems, after completion of three cycles of treatment. Three patients are alive, including one CR patient (25 months) and two SD patients (35 and 20 months). The QOL of PD patients was maintained until the end of the treatment. The Eastern Cooperative Oncology Group (ECOG) performance status at the end of the treatment had not deteriorated.

Total bilirubin of the HCC patients who had received IFN-β and doxorubicin intra-arterial combination therapy decreased significantly after one cycle ($P = 0.0344$) and two cycles ($P = 0.0051$) of treatment (Fig. 4). In all patients, anorexia and lassitude were alleviated, offering remarkable benefits for advanced HCC patients.

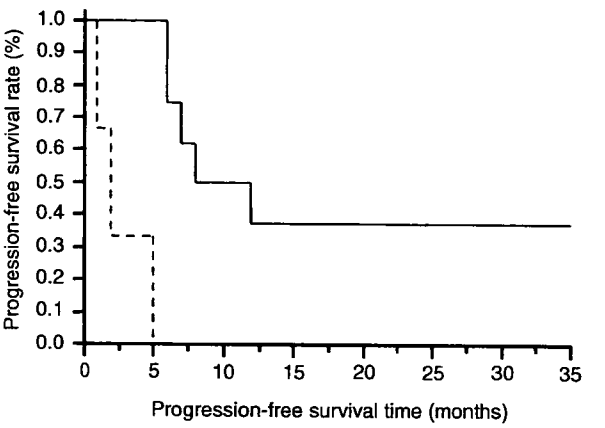

Figure 3 Progression-free survival times of patients with advanced hepatocellular carcinoma according to responses to β-interferon and doxorubicin combination therapy. One-year survival rate for CR or SD patients was 62.5% (5/8) and that for PD was 0% (0/3). The progression-free survival time for CR or SD was longer than that for PD. (——), CR-SD; (- - -), PD. CR, complete response. ($P = 0.0004$, log-rank test).

DISCUSSION

HEPATOCELLULAR CARCINOMAS RECEIVE nourishment from the hepatic artery, not the portal

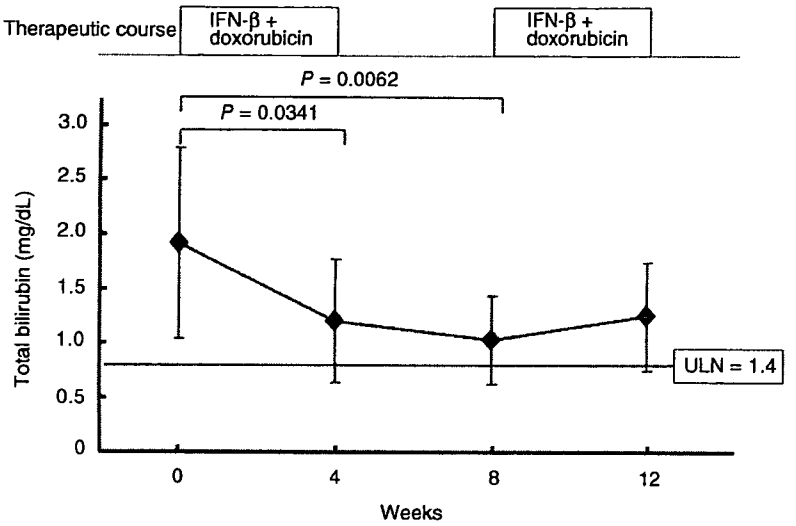

Figure 4 Serum bilirubin ameliorated during combined β-interferon (IFN-β) and doxorubicin intra-arterial injection therapy. Serum levels of total bilirubin decreased significantly and entered the normal range after the first course of combined chemotherapy, and remained in the normal range during the further courses. Values are averages ± SD. Upper limit of normal (ULN) serum values of total bilirubin, 1.4 mg/dL.

flow. Thus, a therapeutic effect should be attainable by giving antitumor agents via the hepatic artery. By direct delivery into the hepatic artery, the concentrations of anticancer agents in the liver increase to 10-fold or more than those by administration via the peripheral veins.[18] By direct injection of anticancer drugs into blood vessels draining to local areas, higher therapeutic effects can be expected when higher ratios of drug concentration appear in the internal organs on their first pass (first-pass effect).[19] When doxorubicin is infused from the hepatic artery, the first-pass effect in the liver is considered to be approximately 60% in rabbits. As the antitumor effects are dose dependent, anthracyclines, including doxorubicin, should be suitable for intra-arterial chemotherapy by single bolus injection.[20] Doxorubicin is metabolized in the liver by hepatic cytochrome P450 and is excreted in bile and urine.[21] On being metabolized by the typical P450 CYP3A4, 40% or more of doxorubicin is ultimately excreted via the bile. Its metabolism and excretion are delayed in patients with hepatic dysfunction such as cirrhosis or with obstructive jaundice, in whom the side-effects of anthracyclines tend to develop easily. In the present study, myelosuppression was observed in two patients (nos. 2 and 6), and in one case G-CSF had to be used. We have examined the concentration of doxorubicin of 10 patients including these patients. The blood concentration of doxorubicin was measured by high-performance liquid chromatography using patients' serum. In two patients with myelosuppression, the blood concentrations of doxorubicin exceeded 10 ng/mL at 60 min after the start of administration. In these patients, no significant hepatic damages were observed. Another eight patients without significant myelosuppression, whose blood concentration of doxorubicin could be measured, showed lower blood concentration than 10 ng/mL. These findings suggested that patients, in whom the blood doxorubicin concentration is 10 ng/mL or more at 60 min after the start of administration, seem to be susceptible to the side-effects, especially hematological toxicity. In general, the serum concentration of doxorubicin at 60 min after its administration is less than 10 ng/mL in normal subjects. But it could be well considered that the serum concentration of doxorubicin at 60 min after its administration to the patients with liver dysfuction is more than 10 ng/mL due to the delayed metabolism and excretion of doxorubicin. The monitoring of serum concentration of doxorubicin seems to be important in patients with liver cirrhosis.

IFN-β and doxorubicin intra-arterial combination therapy significantly reduced total bilirubin, but did not improve other liver function tests such as prothrombin time and albumin, This seems to be the most distinct hallmark of this therapy. In the present study, no patients had tumor thrombus in the bile duct, However, in the cases of advanced HCC, tumors may compress the small bile duct. After the treatment of combination therapy, compression of the small bile duct by tumors may be relieved because of the reduction of tumor size. However, giving IFN to bile duct-ligated rats has been reported to result in significant preservation of histology, inhibition of collagen accumulation and partial

improvement of serum markers of cholestasis.[21] Thus, IFN used with doxorubicin may bring about the partial improvement of cholestasis in patients with advanced HCC. However, the mechanism of reduction of serum bilirubin by this combination chemotherapy remains to be clarified. Marked improvement of total bilirubin by IFN-β and doxorubicin therapy in HCC patients might offer clinical proof of the novel characteristics of interferon.

Yang *et al.* reported the efficacy of gemcitabine and doxorubicin for patients with advanced HCC, with median survival of 4.6 months for all patients and median progression-free survival of 2.5 months.[22] Obi *et al.* reported the efficacy of combination therapy of systemic IFN-α and intra-arterial 5-FU for HCC patients with portal vein invasion, with the survival rate at 12 months being 34% and median survival time of 6.9 months.[23] The 1-year survival rate for CR or SD patients was 62.5% and that for all patients, including PD patients, was 45%, and the mean survival time for all patients was 10 months in the present study, although the number of the patients was small. The present findings suggested that IFN-β is more effective than gemcitabine or IFN-α for advanced HCC. This might explain the effectiveness of IFN-β injected into the tumor site in the liver directly through the catheter. To confirm the superior effects of intra-arterial IFN-β administration, further studies with more patients and longer treatment periods should be done.

All patients enrolled in the present study had extensively advanced HCC, with five cases including portal tumor thrombus Vp3. Patients with Child–Pugh grades A and B are also eligible for this combined chemotherapy regimen, but the dose and the interval of administration should be considered for patients with ascites or a serum level of total bilirubin at 3.0 mg/dL or more, such as Child–Pugh grade C.

Small amounts of IFN-β and doxorubicin do not tend to cause severe side-effects. Under the new enrollment criteria, HCC patients need only 2 or 3 days of hospital stay for port implantation, and outpatient therapy can be started immediately. Moreover, this one-shot intra-arterial injection therapy can be conducted within a short time to minimize restriction of the patient. Based on these findings, one-shot intra-arterial combination chemotherapy of IFN-β and doxorubin could be recommended for outpatient therapy of patients with advanced HCC.

In conclusion, for patients with progressive hepatocellular carcinoma, this preliminary study shows that combined IFN-β and doxorubicin intra-arterial chemotherapy has the potential of prolonging survival time while maintaining QOL in an outpatient clinic. This combination chemotherapy, with tolerable side-effects, has the potential of serving as an optimal treatment option for advanced HCC, by improving liver function and maintaining the QOL for outpatients.

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Original Article

Vitamin K2 inhibits the proliferation of HepG2 cells by up-regulating the transcription of *p21* gene

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Aim: Vitamin K2 has been reported to inhibit the growth of human hepatocellular carcinoma (HCC) *in vitro* and suppress hepatocarcinogenesis *in vivo*. However, its inhibitory mechanism has not yet been clarified.

Methods: Different concentrations of vitamin K2 (30, 10, 1, 0.1 and 0.01 μM) were added to the HCC cell line HepG2 to assess effects on cell growth. The effect of vitamin K2 on cell cycle progression was determined by flow-cytometric analysis. The expression of cell cycle regulatory proteins p21 and p27 was then examined by Western blot. Whether vitamin K2 regulates the gene expression through action on the *p21* promoter region was investigated by luciferase assay.

Results: Vitamin K2 inhibited the growth of HepG2 cells dose-dependently, and its inhibitory rate reached approximately 50% at the dose of 30 μM after 96 h treatment. After treatment with vitamin K2, the proportion of cells in G0–G1 phase increased, and in S phase decreased. Apoptotic cells were not detected. The expression of cell cycle regulatory protein p21 was induced by vitamin K2 treatment, but p27 was not. By the luciferase assay, vitamin K2 significantly activated the promoter of p21. Knock-down of p21 by siRNA reversed the growth inhibition of HepG2 cells by vitamin K2.

Conclusions: The findings suggest that vitamin K2 suppresses the proliferation of HCC cells by blocking the cell cycle G1/S progression through the transcriptional induction of *p21*.

Key words: hepatocellular carcinoma, HepG2, p21, vitamin K2

INTRODUCTION

VITAMIN K, AN essential vitamin, consists of different forms. Vitamin K1 (phylloquinone) is found in green leafy vegetables, vitamin K2 (menaquinone) is produced by the intestinal flora, and other vitamin K congeners such as vitamin K3 (menadione) and vitamin K5 are synthetic.[1,2] Vitamin K and its derivatives have been shown to possess cell growth inhibitory effects on a variety of human and murine cancer cell lines; however, the mechanisms of the inhibitory action have not yet been clarified.[3–7]

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The *in vivo* preventive effect of vitamin K2 on the development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C and its *in vitro* inhibitory effect on the growth and invasiveness of HCC cells have been reported.[3,5,8] Some mechanisms of the inhibitory effect by vitamin K2 have been shown to be via protein kinase A activation[5] or cell cycle arrest.[6,7] The regulation of cell cycle progression was demonstrated at the transition from G1 to S phase and suggested to be the cause of the increased expression of cell cycle regulatory protein(s) or reduced expression of cyclin-dependent kinase 4 (Cdk4). Cell cycle regulatory proteins p21 and p27 are negative regulators of G1/S progression and play important roles in regulating tumor formation and progression in humans.[9–12] The transcription of the *p21* gene is directly activated by wild-type p53 tumor suppressor protein and could play a key role as a downstream mediator of the p53-induced cell growth arrest.[9] Here, we investigated the effect of vitamin K2 on p21 and p27 in HepG2 cells.

METHODS

Materials

VITAMIN K2 (MENATETRENONE, MK-4) was supplied from Eisai (Tokyo, Japan). The human wild-type full-sizes of *p21* and *p27* promoter-luciferase fusion plasmids, pWWP and p27PF, and Sp1 deletion mutant *p21* promoter plasmids, pWP101, pWP-mtSp1-3, pWP-mtSp1-4, and pWP-mtSp1-5-6, were as reported previously.[13–15] p21 Waf1/Cip1 siRNA (Human Specific) kit was purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Human HCC cell line HepG2 was purchased from the American Type Culture Collection (ATCC). HepG2 cells were cultured in Dulbecco's modified Eagle's essential medium (DMEM; Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO_2.

Cell growth assays

Cells were seeded onto 96-well plates at a density of 2.5×10^3 cells. After 20 h culture, 100 μL of fresh medium containing different concentrations of vitamin K2 (30, 10, 1, 0.1 and 0.01 μM) was added to each well. Vitamin K2 was dissolved in 99% ethanol at the concentration of 10 mM, and then diluted with DMEM to the appropriate concentrations for experiments. Control cells were cultured in DMEM containing the corresponding concentration of ethanol to each dose of vitamin K2.

After 2-, 3- or 4-day culture with vitamin K2 treatment, the number of viable cells in each well was determined with the 3-(4,5-dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide) (MTT) assay (Roche, Nutley, NJ, USA) according to the manufacturer's instructions.[16]

For *p21* siRNA assay, 50 and 100 nM of *p21* siRNA were transfected to the HepG2 cells for 24 h, then 30 μM of vitamin K2 was added and the cells incubated for 96 h.

All experiments were carried out four times concurrently, and then repeated three times.

Cell cycle

Cell cycle distribution was determined by flow cytometric analysis of DNA content (Becton Dickinson, San Jose, CA, USA) after 96 h treatment of vitamin K2 at 10 or 30 μM. Cell suspensions were fixed overnight in 2 mL of 70% ice-cold ethanol and incubated with RNase at a concentration of 0.25 mg/mL at 37°C for 1 h. Cells were treated with propidium iodide (50 μg/mL) for 30 min in the dark. DNA histograms were analyzed using Lysis-II software (Becton Dickinson) to evaluate the cell cycle components.[16]

Western blotting

After 96 h culture with vitamin K2, HepG2 cells were washed twice with ice-cold phosphate-buffered saline (PBS), lysed and sonicated in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL phenylmethylsulfonyl fluoride, 45 μg/mL aprotinin, 100 mM sodium orthovanadate). The supernatant of the homogenate was used for protein determination with a BCA Protein Assay Kit (Pierce, IL, USA) and electrophoresis. Samples with equal amounts of total protein were electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing conditions and blotted to polyvinylidene difluoride (PVDF) membrane by electroblotting. Actin was also used to confirm equal loading. The membranes were blotted with anti-p21 antibody or anti-p27 antibody.[16] Signals were developed with an ABC kit (Vector, Burlingame, CA, USA) and diaminobenzidine. The densities of the immunoreactive bands of p21 and p27 protein were estimated by NIH Image software.

Luciferase assay

HepG2 cells (5×10^4 cells/dish) were seeded in 35 mm culture dish (Falcon, Lincoln Park, NJ, USA) in phenol red-free DMEM containing with 5% charcoal-dextran-stripped fetal bovine serum (FBS-CCS). The cells were transfected with 0.5 μg p21 or 2.5 μg p27 reporter vectors using a calcium-phosphate transfection kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Twenty-four hours later, culture media were changed to the fresh media with several concentrations (0, 10 and 30 μM) of vitamin K2, and incubated for 24 h. After incubation for 24 h, cells were harvested and lyzed with luciferase lysis buffer (Promega, Madison, WI, USA). Proteins were measured by BCA protein assay kit. Luciferase activity of each sample was measured by luciferase assay kit (Promega). The level of induction was calculated by dividing the mean luciferase activity of samples treated with vitamin K2 by the mean activity of untreated control samples. All experiments were carried out in triplicate and repeated at least three times.

Statistical analysis

The results are expressed as means ± SE. At least three separate experiments were performed for each data

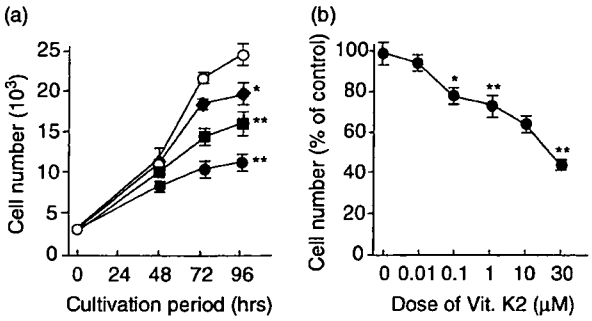

Figure 1 Vitamin K2 suppressed the proliferation of HepG2 cells in a dose-dependent manner. HepG2 cells were treated with various doses of vitamin K2 or the medium containing ethanol corresponding to 30 μM of vitamin K2 as the control. (a) Proliferation curves of HepG2 cells at various doses of vitamin K2 (1, 10 and 30 μM) for 48–96 h. ○, 0 μM; ◆, 1 μM; ■, 10 μM; ●, 30 μM. (b) Dose-dependent inhibition after vitamin K2 treatment for 96 h. Data are shown as mean ± SE of three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ *vs* control.

point. Statistical analyses were done using Student's unpaired *t*-test (two-tailed) or chi-squared test.

RESULTS

Effect of vitamin K2 on HepG2 growth

VITAMIN K2 SHOWED a dose-dependent inhibition on the proliferation of HepG2 cells (Fig. 1). The inhibitory effects by vitamin K2 after 96 h treatment were 53% and 32% at 30 μM and 10 μM, respectively. Even at 1 μM, vitamin K2 significantly inhibited the growth of HepG2 cells by about 20% after 96 h treatment ($P < 0.05$).

Effect of vitamin K2 on cell cycle progression

The cell cycle distribution of HepG2 cells was analyzed by flow cytometer after 4 days treatment with vitamin K2 (Fig. 2). The proportion of HepG2 cells in G0–G1, S and G2-M phases of the cell cycle were 58.5 ± 0.8%, 30.7 ± 1.6% and 10.9 ± 1.6%, respectively, with no treatment. After incubation with vitamin K2 for 96 h, the proportion of HepG2 cells in G0–G1 changed to 67.8 ± 1.7% and 72.8 ± 1.6%, and those in S phase to 21.0 ± 1.1% and 16.4 ± 2.7%, at 10 and 30 μM, respectively. The proportion of cells in G0–G1 phase increased, and those in S phase decreased significantly, as compared to the cells with no treatment ($P < 0.01$). In contrast, the proportion of cells in G2-M phase were 11.3 ± 2.7% and 10.9 ± 1.5% after vitamin K2 treatment at 10 and 30 μM, respectively, indicating that vitamin K2 did not affect the cell cycle phase from G2 to M. Apoptotic cells were not detected. These results showed that vitamin K2 inhibited the transition from G1 into S phase, resulting in G1 arrest.

Effect of vitamin K2 on expression of p21 and p27

To determine the molecular mechanism inducing G1 arrest by vitamin K2, the expression of cell cycle regulatory proteins p21 and p27 was examined by western blot. After 96 h treatment, p21 protein expression increased 1.77 ± 0.15 and 3.01 ± 0.16 fold by relative density at 10 and 30 μM of vitamin K2, respectively ($P < 0.01$). In contrast, p27 protein expression did not significantly increase (1.04 ± 0.09 at 10 μM and 0.93 ± 0.12 fold at 30 μM of vitamin K2; Fig. 3).

Effect of vitamin K2 on the activation of *p21* and *p27* promoters

Next, we investigated whether vitamin K2 regulates the gene expression through the action on the *p21* promoter region by use of the luciferase assay. The *p21* and *p27* reporter plasmids pWWP and p27PF were transiently transfected in HepG2 cells, and luciferase activities were examined. In HepG2 cells transfected with pWWP, the relative luciferase activity increased to 3.19 ± 0.32 and 6.04 ± 0.44 fold over than the control after vitamin K2 treatment of 10 and 30 μM, respectively (Fig. 4a). The relative luciferase activity did not increase in HepG2 cells transfected with p27PF (1.03 ± 0.11 fold at 10 μM and 0.9 ± 0.17 fold at 30 μM; Fig. 4b).

We further investigated the action sites of vitamin K2 on the promoter region of *p21* gene using several Sp1 deletion mutants of *p21* promoter. The luciferase activities did not significantly increase after the pWP101, pWP-mtSp1-3, pWP-mtSp1-4, and pWP-mtSp1-5-6 vectors were transfected (Fig. 4c). These results showed that four Sp1 sites between −101 to 0 in the *p21* promoter were not necessary, but the sequence between −2320 to −102 region was responsible for the induction of p21 by vitamin K2.

Effect of knock-down of p21 by siRNA on vitamin K2-induced growth inhibition

Next, we investigated whether the inhibition of p21 induction reversed the effect of vitamin K2 on HepG2 cells. The expression of p21 protein was suppressed after *p21* siRNA transfection (data not shown). After the knock-down of p21 by 50 nM and 100 nM *p21* siRNA,

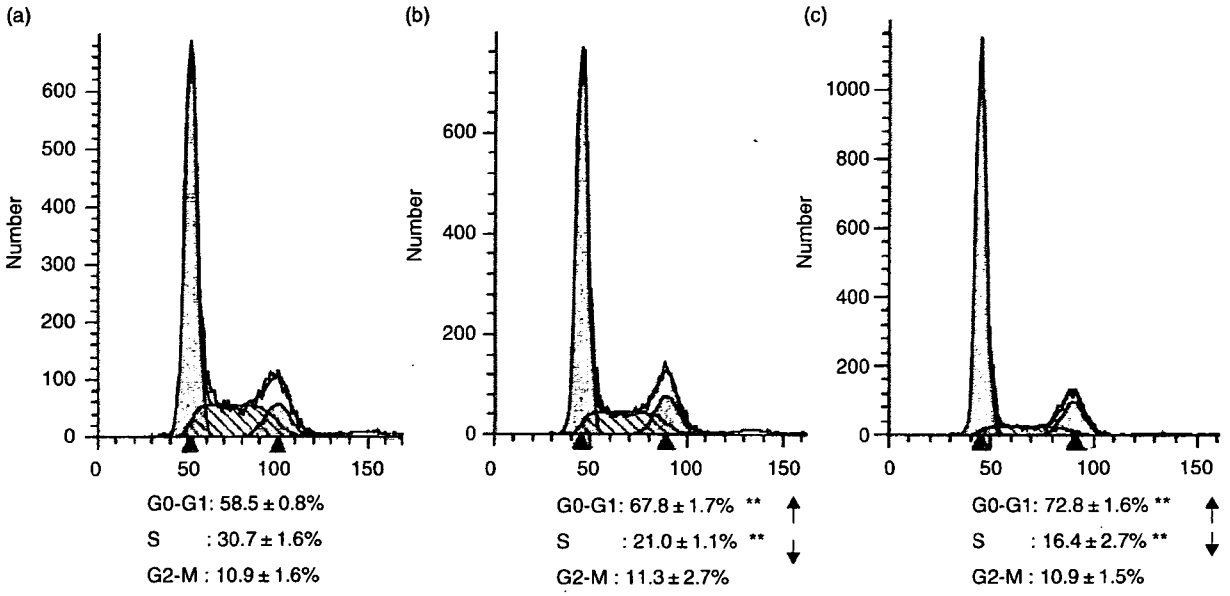

Figure 2 Effects of vitamin K2 on cell cycle progression. Cell cycle distribution was determined by flow cytometric analysis after 96 h treatment of vitamin K2. Each DNA histogram is a representative of three independent experiments at (a) 0, (b) 10 and (c) 30 μM of vitamin K2. The percentages of G0–G1, S and G2-M phase cells at each panel are means ± SE of three independent experiments. $^{**}P < 0.01$ *vs* control.

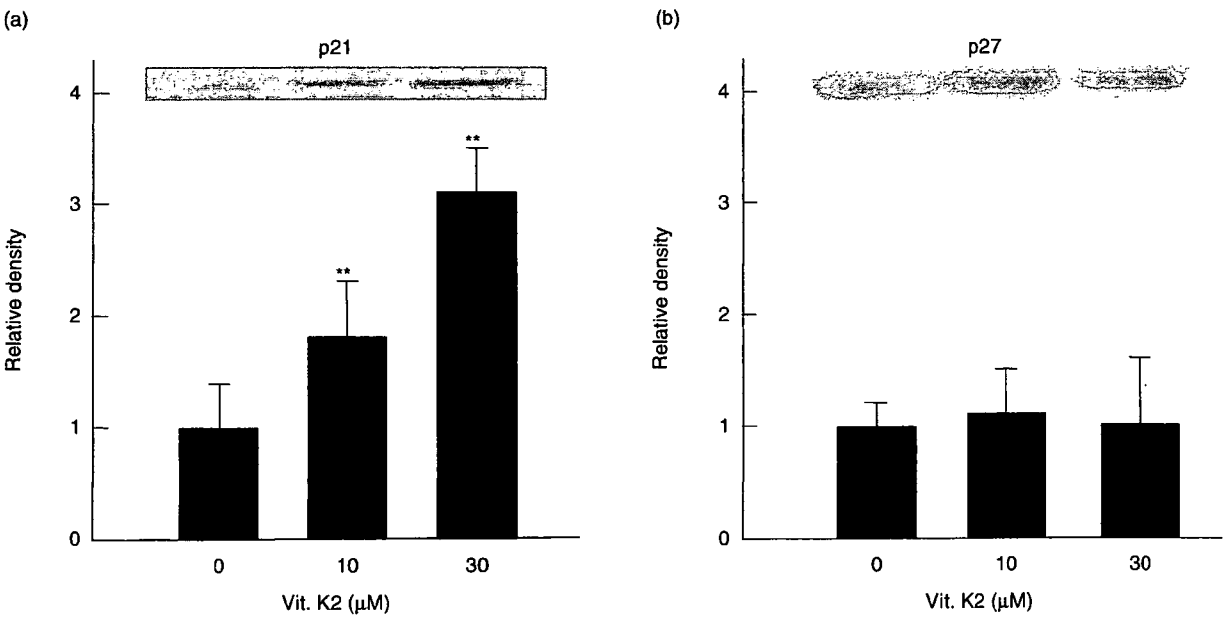

Figure 3 Vitamin K2 induced the expression of p21 protein, but not p27 protein. Expression of (a) p21 protein and (b) p27 protein was evaluated by western blot after 96 h treatment of vitamin K2 at 0, 10 and 30 μM, and the densities of immunoreactive bands were measured by NIH Image software. Data are shown as means (bars, SE) of three independent experiments. $^{**}P < 0.01$ *vs* control.

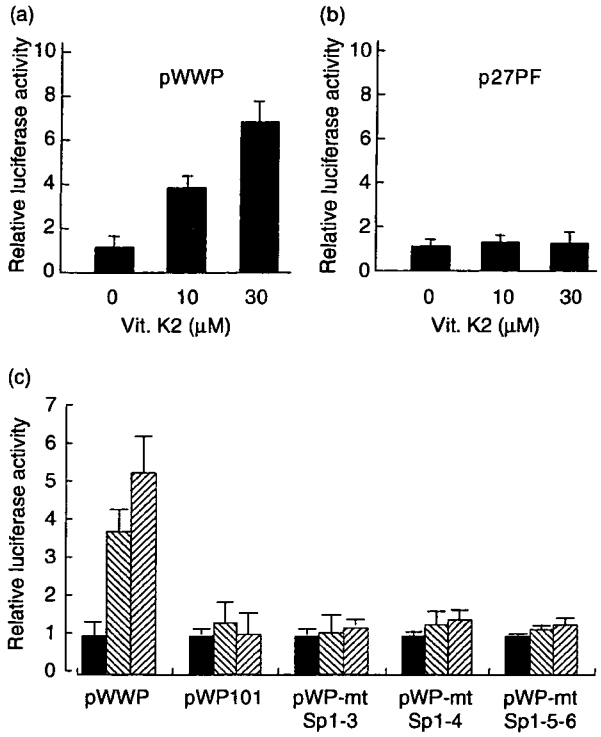

Figure 4 Vitamin K2 activated the p21 gene promoter, but not p27 gene promoter. pWWP and Sp1 deletion mutant vectors were used as luciferase reporter plasmids for p21 gene promoter, and p27PF was used for p27 gene promoter. HepG2 cells were transfected with 0.5 μg of p21 or 2.5 μg of p27 promoter vectors. After incubation with indicated concentrations of vitamin K2 for 24 h, cells were harvested and relative luciferase activities were measured. (a) pWWP, (b) p27PF and (c) Sp1 deletion mutant p21 vectors. ■, 0 μM; ▨, 10 μM; ▨, 30 μM.
Data are shown as means (bars, SE) of three independent experiments.

the cell growth inhibited by vitamin K2 at 30 μM recovered from 46.9 ± 1.8% to 54.9 ± 2.8% and 73.0 ± 9.1%, respectively (Fig. 5). These findings suggest that p21 is necessary for the growth inhibition of HepG2 cells by vitamin K2.

DISCUSSION

VITAMIN K2 HAS been reported to inhibit the growth of HepG2 cells in dose-dependent manner.[5,6] The inhibitory mechanism of vitamin K2 has been shown to cause the arrest of cell cycle progression.[7,17,18] By FACScan method, the G1–S block has been reported to be induced by vitamin K2 treatment in HepG2 cells.[3,6] The present results demonstrating the growth inhibition through G1 arrest induced by vitamin K2 are consistent with these previous observations.

In the eukaryotic cell cycle, several positive and negative factors regulate cell cycle progression.[9] Among the positive cell cycle regulators, the key players are a family of protein kinases termed cyclin-depedent kinase (Cdk). Cdks play important roles in promoting the transition from G1 to S phase by the phosphorylation of the retinoblastoma protein (pRB).[9] The negative cell cycle regulators, of which the important proteins are p21 and p27, appear to function as broad specific inhibitors of Cdk complexes.[9] Vitamin K2 has been reported to inhibit the growth of HCC cell line PLC/PRF/5 by inducing Cdk4.[17] Overexpression of p21 has been reported to suppress the growth of MG63 osteosarcoma cells and induce the differentiation of carcinoma cells.[18] p21 and p27 also play important roles in cell-cycle arrest induced by cyclooxygenase-2 inhibitor (etodolac) or PPAR-γ ligand (troglitazone) in human hepatoma cells.[15,16] The p21 expression was reported to be decreased in HCC.[19] The increased expression of p21 after vitamin K2 treatment has been shown by immunoblot in HepG2 cells, but at a higher concentration (500 μM) than the clinically used dose.[6] In this study, we confirmed the induction of p21 protein in HepG2 cells by vitamin K2 at the concentration of clinically used dose (30 μM), by Western blot, and furthermore the induction of *p21* gene by luciferase promoter assay. In addition, the suppression of vitamin K2-induced p21 by siRNA reversed the growth inhibitory effects of vitamin K2. These results

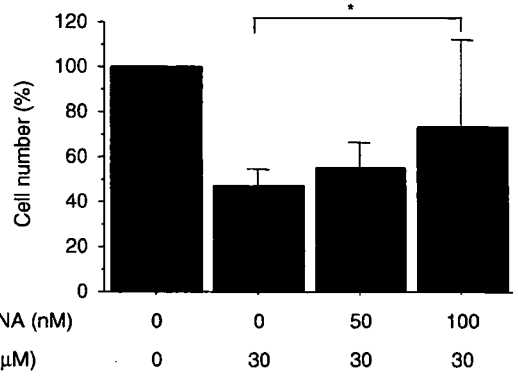

Figure 5 Knock-down of p21 by siRNA reversed the growth inhibitory effects of vitamin K2. p21 siRNA was transfected to HepG2 cells at the concentration of 50 and 100 nM. Twenty-four hours later, vitamin K2 (30 μM) was added and incubated for 96 h. Cell numbers (%) are shown as means (bars, SE) of three independent experiments. $^*P < 0.05$.

show that p21 plays a key role in the growth inhibition of HepG2 cells by vitamin K2. However, the induction of p27 was not observed by either western blot or luciferase reporter assay. No previous study has reported on the induction of p27 by vitamin K2. Thus, p27 seems to play no important roles on the growth inhibition of HCC induced by vitamin K2. These findings suggest that vitamin K2 suppresses the proliferation of HepG2 cells by the mechanism of G1 arrest via the induction p21 cell cycle regulator through the activation of *p21* gene promoter.

p21 was thought to be a target gene of vitamin K2 and vitamin D3.[18] Some medications such as butyrate, trichostatin A and vesnarione have been reported to directly bind to the promoter region and activate the transcription of *p21* through Sp1 sites in several cancer cell lines.[11,12,20] The Sp1 site is also the binding site of vitamin D3 in the *p27* gene promoter.[15] In the present study, the Sp1 site in −101 to 0 region of *p21* promoter was not responsible for vitamin K2 action. The upstream region from −102 to −2320 was essential for p21 induction by vitamin K2. Thus, the Sp1 site seems to be not necessary for interaction with vitamin K2. It is very important to analyze the detailed mechanism of the action of vitamin K2 on the *p21* promoter in future.

In conclusion, our results indicate that vitamin K2 activates the promoter region and enhances the transcription of *p21* gene, which then suppresses the proliferation of HCC cells. Although future study is still needed to investigate the detailed mechanism of the effect of vitamin K2 on cell growth inhibition, these findings suggest that vitamin K2 might be a useful treatment for HCC.

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A Phase I Study of Combination Therapy of the Oral Fluorinated Pyrimidine Compound S-1 with Low-dose Cisplatin Twice-a-week Administration (JFMC27-9902 Step2) in Patients with Advanced Gastric Cancer Using a Continual Reassessment Method

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Objective: We conducted a Phase I study to evaluate the safety and efficacy of a combination of S-1 with semi-weekly low-dose cisplatin in patients with unresectable/recurrent gastric cancer to determine the recommended dose (RD) for a subsequent Phase II study.
Methods: S-1 was administered orally at 80–120 mg/body/day based on body surface area. One cycle consisted of the consecutive administration of S-1 for 28 days followed by 14 days rest. Three dose levels, 7.5, 10, and 15 mg/m^2/day, were set for cisplatin, which was administered twice-a-week for 4 weeks followed by 2 weeks of rest in each cycle. Dose-limiting toxicity (DLT) data were continually monitored to enable decisions regarding cisplatin dose escalation and deescalation based on a new dose-finding algorithm using a continual reassessment method (CRM). The CRM target toxicity level to estimate the RD was set at 20%.
Results: Eight and five patients were treated at cisplatin dose levels of 10 and 15 mg/m^2/day, respectively. Two DLTs occurred at both dose levels. On the basis of this data, the CRM estimated the RD to be 10 mg/m^2/day of cisplatin. Three patients of eight patients treated with 10 mg/m^2/day of cisplatin exhibited a confirmed partial response during the treatment period.
Conclusion: For future trials examining the safety and efficacy of daily S-1 with semi-weekly cisplatin in patients with unresectable/recurrent gastric cancer, we found a cisplatin RD of 10 mg/m^2/day.

Key words: S-1 – low-dose cisplatin – continual reassessment method – gastric cancer – Phase I clinical study

INTRODUCTION

The oral dihydropyrimidine dehydrogenase inhibiting fluoropyrimidine S-1 is both safer and more effective than other therapies for the treatment of recurrent and/or unresectable gastric cancer (1–3), and S-1 has been incorporated into standard gastric cancer treatment regimens in Japan. A variety of S-1 based combination chemotherapies have been studied to establish a more effective treatment regimen that minimizes the occurrence of adverse events. In particular, combinations of cisplatin (CDDP) (4–8), docetaxel (9,10), paclitaxel (11), or irinotecan (12) with S-1 have shown promise for the treatment of gastric cancer through Phase

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I or II study. Two regimens combining different doses of CDDP with S-1 were examined: a high-dose regimen with 60 mg/m^2 CDDP once in 5 weeks (7) or 75 mg/m^2 once in 4 weeks (8) or a low-dose regimen involving weekly 25 mg/m^2 or less administration of CDDP (4–6). Additionally, Hyodo et al. (4) conducted a dose-finding Phase I study and determined that weekly administration of 20 mg/m^2 CDDP was optimal when combined with 70 mg/m^2 of S-1 administered daily for 2 weeks followed by a 1-week washout period. With this regimen, 58% of patients responded to therapy, but 54% of patients experienced Grade 2 gastrointestinal toxicity during the first two cycles. This is particularly concerning because oral anticancer agents such as S-1 must achieve high concentrations to be maximally effective, and gastrointestinal toxicity including nausea and anorexia severely limits this. A lower dose of CDDP administered more frequently may be as effective with less gastrointestinal toxicity.

The Japanese Foundation for Multidisciplinary Treatment of Cancer carried out a Phase I clinical trial (JFMC27-9902) examining the safety of escalating low doses of CDDP given in combination with a fixed dose of S-1 five times per week (5). Among the doses of CDDP examined, 1, 2, 3, 4, or 6 mg/m^2, 4 mg/m^2 given 5 days per week was optimal. However, the dosing schedule would require a large degree of hospitalization that is not compatible with the current medical and economic environment in Japan. The reality of this situation suggests that a new Phase I/II study is needed to determine an optimal twice-a-week CDDP administration schedule that maintained or increased regimen efficacy while minimizing adverse events (13). Data from the previous Phase I trial was used as a baseline for the development of a twice-a-week CDDP dosing schedule in the current Phase I study (JFMC27-9902 Step2), and we adopted a new dose-finding algorithm using a continual reassessment method (CRM) (14,15) to estimate a recommended dose (RD) for the new dosing schedule. Data from the present study will be used to develop a Phase II study to examine the efficacy of a novel CDDP, S-1 combination regimen for the treatment of unresectable and/or recurrent gastric cancer.

PATIENTS AND METHODS

Trial Eligibility

Patients with a histologic diagnosis of unresectable or recurrent gastric cancer and a performance status (PS) of Eastern Cooperative Oncology Group of 0 to 1 were eligible for study participation. Additional eligibility criteria included: (i) age ranging from 20 to 75 years, (ii) no anti-tumor therapy within 28 days prior to enrollment except for hormone-therapy and immunotherapy completed 2 weeks before enrollment into this trial and postoperative adjuvant chemotherapy not using CDDP completed 1 month before enrollment into this trial, (iii) life expectancy longer than 12 weeks, (iv) adequate bone marrow function (Hb $\geq$9.0 g/dl, white blood cells between 4000 and 12 000/μl, platelets $\geq$100 000/μl), and (v) sufficient organ function (total bilirubin $\leq$1.5 mg/dl, GOT and GPT $\leq$2.5 times the upper normal level, alkaline phosphatase $\leq$ two times the upper normal level, and blood urea nitrogen (BUN) and serum creatinine $\leq$ the upper normal level). Informed consent was obtained from each patient before enrollment. Each institutional review board for human experimentation approved the protocol of this study.

Treatment Regimen

S-1 was administered orally twice daily after a meal at one of three initial doses based on body surface area (BSA): (i) BSA $<$1.25 m^2, 80 mg per day, (ii) 1.25 m^2 $\leq$ BSA $<$ 1.5 m^2, 100 mg day per day, and (iii) 1.5 m^2 $\leq$ BSA, 120 mg per day. One cycle consisted of twice daily S-1 administration for 28 consecutive days followed by 14 days of withdrawal. CDDP in 100 ml of normal saline was given as a 1-h intravenous injection twice a week for 4 weeks on days 1, 4, 8, 11, 15, 18, 22, and 25, followed by 2 weeks of withdrawal in each cycle. Three CDDP dose levels, 7.5 mg/m^2 per day (Level 1), 10 mg/m^2 per day [Level 2; starting dose (see below)], and 15 mg/m^2 per day (Level 3), were chosen. These doses were determined using data from the previously conducted Phase I trial JFMC27-9902 (5). No patient was given hydration to protect against nephrotoxicity.

Patients underwent at least two consecutive cycles of combination therapy. Patients remained in the study unless (i) dose-limiting toxicities (DLTs) (within the first cycle) or Grade 4 hematological or Grade 3 or 4 non-hematological toxicities (after the first cycle) occurred, (ii) objective evidence of tumor progression appeared, or (iii) the patient refused to continue the treatment. Additionally, therapy was discontinued if hematologic toxicities including Grade 2 or greater leukopenia, neutropenia and thrombocytopenia, Grade 2 or greater non-hematologic toxicities (except alopecia), and a deterioration in PS of two or more during each treatment cycle were observed. If the toxicity causing treatment discontinuation was Grade 3 or greater leukopenia, neutropenia or Grade 2 or greater thrombocytopenia, the S-1 dose was reduced from 80, 100, and 120 mg per day to 50, 80, and 80–100 mg per day, respectively. The dose of CDDP was not modified during the first and second cycles.

Assessments of Toxicity and Efficacy

Adverse events were evaluated according to the National Cancer Institute— Common Toxicity Criteria version 2.0. DLT was defined for this study as the occurrence of any of the following observed within the first cycle of treatment: (i) Grade 3 or 4 leukopenia for 3 days or more, (ii) Grade 3 or 4 neutropenia along with fever (febrile neutropenia), (iii) Grade 3 or 4 thrombocytopenia, (iv) Grade 3 or greater non-hematologic toxicity, excluding alopecia, nausea/vomiting, and general fatigue, (v) total treatment interruption lasting $>$3 weeks, or (vi) patient's refusal to continue treatment due to adverse events or related matters. The assessment of

tumor response was based on the RECIST criteria. In addition, the tumor response data were reviewed extramurally. The protocol was approved by the Protocol Review Committee of the Japanese Foundation for Multidisciplinary Treatment of Cancer (JFMC).

Study Design and Statistical Analyses

The RD was estimated using a CRM proposed by O'Quigley and Shen (15). We adapted this approach, because a CRM defines the RD more precisely than a conventional '3 + 3' cohort design, thus the CRM was considered to suit the objective of this study. Dose escalations and deescalations for consecutive patient cohorts and the size of each cohort were based on the dose-finding CRM algorithm and clinical judgment. Skipping from Levels 1 to 3 was not allowed in the CRM calculations. The target toxicity level of the CRM to estimate the RD was set at 20%, which is the minimum value typically used in Phase I trials (15). Prior to starting the trial, participating clinicians predicted possible DLT occurrence probabilities of dose levels 1, 2, and 3 as 10% (5–30%), 20% (10–40%), and 40% (20–70%), respectively, based on the previous Phase I trial (JFMC27-9902) data (5). The ranges in the parentheses represent the pretrial clinician uncertainty of the DLT occurrence probability at each of the three dose levels. We determined the starting dose level in this trial to be Level 2, with the first two enrolled patients (first patient cohort) treated at this level. According to the pre-specified dose-escalation rule, if no DLT was observed in the first two patients, one patient enrolled in the second cohort was treated at Level 3. The projected sample size for the Phase I study was expected to require 10–16 patients, taking the simulation studies performed by O'Quigley et al. (14) into account.

In the CRM calculations, sensitivity analysis for parameters in the dose-toxicity model was performed. Four clinical scenarios for DLT occurrence probabilities at the three dose levels were established based on the pretrial prediction of the probabilities by the clinicians. We used scenarios (i) 10, 20, and 40%, (ii) 5, 10, and 20%, (iii) 30, 40, and 70%, and (iv) 5, 30, and 60% for DLT occurrence probabilities at Levels 1, 2, and 3, respectively. We considered bringing the Phase I to an early close when clear separation of the confidence intervals for the three dose levels appeared. This decision was also made according to clinical judgment. In addition, the trial was designed to be halted at the end of the Phase I if the selected regimen at the RD was insufficiently active when the hypothesis that the response rate (RR) at the RD is over 60% was statistically rejected (16). The Independent Data and Safety Monitoring Committee (IDSMC) independently reviewed the interim analysis and monitored protocol compliance, safety, and on-schedule study progress. The IDSMC considered stopping the trial from clinical as well as statistical points of view.

RESULTS

Patient Characteristics

A total of 13 patients were enrolled from the three sites (Osaka City University Hospital, Kochi Health Sciences Center, and Tohoku Employees' Pension Welfare Hospital) from December 2003 to March 2006. Eight and five patients were treated at Levels 2 and 3, respectively, and their baseline clinical characteristics are summarized in Table 1. The total number of cycles administered ranged from one to five and one to four for patients treated at Levels 2 and 3, respectively. Treatment was discontinued in two patients at Level 2 and two patients at Level 3 during the first cycle of therapy due to the occurrence of DLT. Treatment was discontinued in two patients at Level 2 and one patient at Level 3 during the second cycle, caused by the progression disease. Treatment was discontinued in three and two patients at Levels 2 and 3, respectively, due to the initiation of subsequent therapy. One patient at Level 2 underwent surgery after obtaining a partial response within the second cycle.

Toxicity

Hematologic and non-hematologic toxicities for the 13 patients observed during the first cycle are detailed in Table 2. Grade 3/4 toxicities were observed more frequently in patients treated at Level 3. Two patients treated at Level 3

Table 1. Patient characteristics

	Level 2 ($n = 8$)	Level 3 ($n = 5$)
Gender		
Male	6	4
Female	2	1
Age		
30–49	1	1
50–59	2	0
60–69	4	3
70–75	1	1
Performance status (baseline)		
0	6	4
1	2	1
Diagnosis		
Unresectable	7	5
Recurrent	1	0
Hepatic metastasis		
Negative	1	4
Positive	7	1
Lymph node metastasis		
Negative	3	0
Positive	5	5

Table 2. Hematological and non-hematological toxicities

Adverse event	Level 2 ($n = 8$)				Level 3 ($n = 5$)			
	Grade				Grade			
	1	2	3	4	1	2	3	4
Hematological								
Leukopenia	1	2	0	0	0	1	1	0
Neutropenia	1	2	1	0	0	1	1	0
Anemia	2	3	0	0	3	2	0	0
Thrombocytopenia	4	1	0	0	2	0	0	0
Non-hematological								
Anorexia	2	0	0	0	1	1	2	0
Nausea	0	0	0	0	0	1	1	0
Vomiting	0	1	0	0	0	0	0	0
Diarrhea	0	0	0	0	1	0	0	0
Hand-foot skin reaction	0	0	0	0	1	0	0	0
Fatigue	1	0	0	0	3	0	0	0
ALT/AST	2	1	0	0	2	0	0	0
Creatinine	0	1	0	0	0	0	0	0

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

suffered Grade 3 anorexia during the first cycle, causing the two DLTs observed on Level 3 therapy. Grade 2 gastrointestinal toxicity (vomiting) was observed in only one patient (12.5%) at Level 2. Additionally, one patient suffered Grade 2 anorexia in the second cycle (data not shown). Therefore, two (25%) out of the eight patients at Level 2 experienced Grade 2 gastrointestinal toxicities through the completion of the first and the second cycle.

Dose Escalation/Deescalation

The first two patients treated at Level 2 did not experience any DLT. According to the pre-specified dose-escalation rules of this study, the dose level for the second patient cohort was escalated to Level 3. Although the one patient in the second cohort did not experience any DLT, one of two patients in the third cohort experienced DLT (anorexia, Grade 3). At this point, the CRM was invoked and estimated the DLT occurrence probabilities as 3.1, 8.7, and 24.9% at Levels 1, 2, and 3, respectively. Thus, in the forth cohort, one patient was still assigned to Level 3, and this patient did not experience any DLT. The next single patient enrolled in the fifth cohort suffered DLT (anorexia, Grade 3), and the recalculated DLT occurrence probabilities estimated using the CRM were 5.9, 13.8, and 32.4% at Levels 1, 2, and 3, respectively. Consequently, the estimated Level 3 DLT probability considerably exceeded the target level, and the next patient cohort was treated at Level 2. In the subsequent six patients treated at Level 2, two DLTs (total treatment

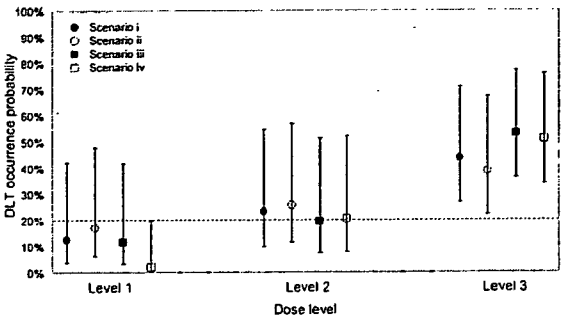

Figure 1. The probabilities of dose-limiting toxicity occurrence with 90% confidence intervals estimated at Levels 1, 2, and 3, based on dose-limiting toxicity data observed the 13 patients. These estimates were performed using the following four clinical scenarios: (i) 10, 20, and 40%, (ii) 5, 10, and 20%, (iii) 30, 40, and 70%, and (iv) 5, 30, and 60%, for DLT occurrence probabilities *a priori* predicted for Levels 1, 2, and 3, respectively.

interruption lasting >3 weeks, patient's refusal to continue treatment due to Grade 2 toxicity) were observed. Figure 1 shows the probabilities of DLT occurrence at Levels 2 and 3, estimated using all DLT data observed in the 13 patients under the 4 clinical scenarios used for sensitivity analysis. When the results of the sensitivity analysis and the observed toxicities at each dose level were considered, the IDSMC suggested that combination therapy at Level 2 was acceptable in terms of safety.

Response to Treatment

The clinical responses of nine patients who did not experience any DLT on the combination therapy were assessed (Table 3). Three patients treated at Level 2 responded to treatment during the Phase I study, and they all exhibited a confirmed PR during treatment. The upper bound of the 95% confidence interval of the RR at Level 2 was 75.5%. Thus, the hypothesis that the RR at the RD is over 60% was not rejected, and these data indicate that the combination therapy regimen is effective and supports proceeding to the Phase II trial.

DISCUSSION

We conducted a dose-finding Phase I trial in 13 patients with unresectable or recurrent gastric cancer to determine the RD of a regimen combining S-1 and low-dose CDDP with twice-a-week administration (JFMC27-9902 Step2). We found that the intensity of CDDP per week at the RD level

Table 3. Tumor response

Dose level	CR	PR	SD	PD	NE
2	0	3	0	3	2
3	0	0	1	2	2

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, non-evaluable.