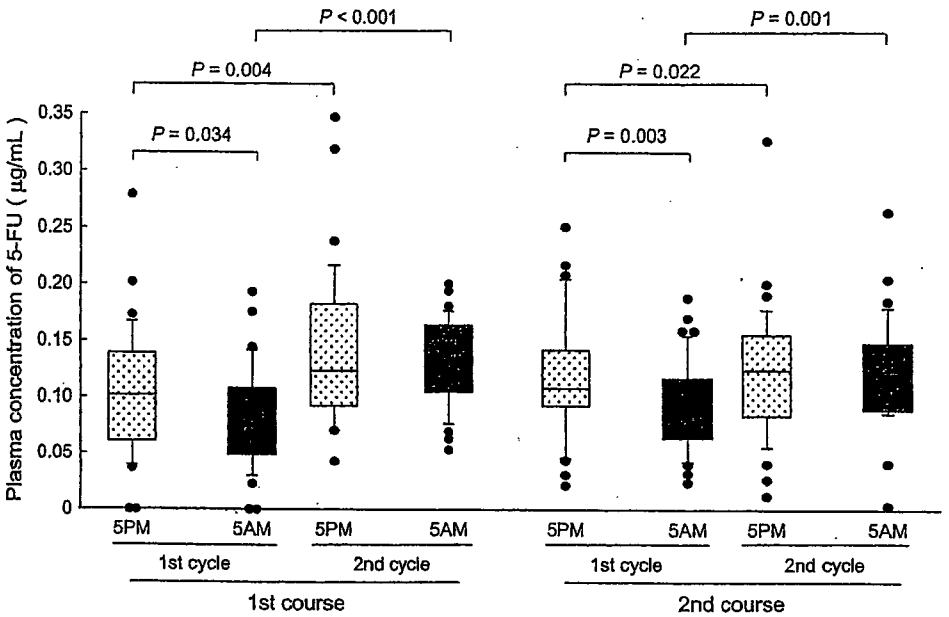

FIGURE 2. Plasma concentrations of 5-FU during repetitive PVI in 30 patients with esophageal cancer. PVI, protracted venous infusion. Comparisons between plasma concentrations of 5-FU at 5 PM and 5 AM were performed by means of Wilcoxon signed-rank test.

with the variant C-allele had a tendency for smaller CV values compared with those without the C-allele.

DISCUSSION

For the nonsurgical treatment of esophageal cancer, the present 5-FU-based chemoradiotherapy including weekly repeated PVI of 5-FU has been accepted, and the evidence supporting its clinical efficacy has been collected by Ohtsu and co-workers during the last decade in Japan.[3,12,13] The Ohtsu regimen is still open to discussion on the dose setting of 5-FU, but the pharmacological data are not available. Because 5-FU may have severe adverse effects such as myelosuppression, mucositis, and diarrhea, the importance of monitoring the plasma concentration of 5-FU has been discussed for safety and effectiveness. Yet there is little information about the therapeutic window of 5-FU in esophageal cancer.[16] As one possible cause of this, the large intra- and interpatient variability of plasma concentration of 5-FU has been considered in head and neck cancer, which is often treated using similar regimens to those of esophageal cancer.[27–29] 5-FU is a typical drug showing a circadian variation of its plasma concentration during PVI, which indicates that sampling time has a large influence on the interpretation of the drug concentration obtained.[19,30]

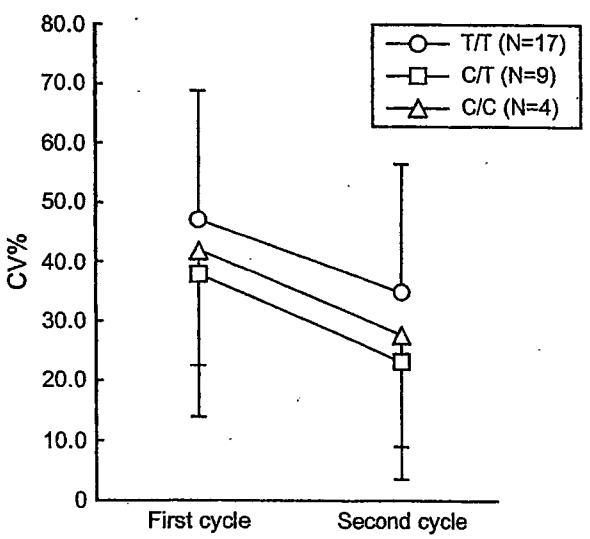

FIGURE 3. Effect of *T3111C* polymorphism on CV values of plasma concentrations of 5-FU in first and second cycles. The coefficient of variation (CV) was calculated as the ratio of standard deviation to the mean value (CV% = 100 × SD/mean). Open circles, T/T (n = 17), open squares, T/C (n = 9); open triangles, C/C (n = 4). Each point represents the average and standard deviation of the respective CV values in each cycle through the first and second courses for 3 genotype groups. There was no significant difference in CV values at any cycles.

Reportedly, 5-FU is rapidly eliminated with a primary half-life between 4 and 25 minutes,[31] which indicates that this drug can achieve steady state within 1 day at the most. In the present study, blood samplings at steady state were performed at 5 PM on day 3 and 5 AM on day 4 after the start of PVI of 5-FU. These points lie in the middle of the duration of PVI. The Ohtsu regimen, used in the present study, includes 4 identical schedules of PVI of 5-FU (Fig. 1). This regimen has the advantage of evaluating the reproducibility of the plasma drug concentration and its variations between active and inactive phase, cycle to cycle, and course to course. Therefore, the data obtained in the present study were analyzed from the following 3 different angles.

First, analyzed according to phase of activity, there was a significant change in the plasma concentrations between active and inactive phases, with higher levels in active phase compared with inactive phase (Fig. 2). This is possibly because of the circadian rhythm with daily active and inactive phase as reported previously.[18,19] Different studies showed different results in the times of peak and trough plasma concentrations,

using a cosinor curve of plasma concentrations, and peak and trough were observed alternately every 12 hours.[18,19,30,32] From these reports, our sampling method every 12 hours was reasonable for analyses of fluctuating patterns.

Second, analyzed according to cycle, drug concentrations in the second cycles were significantly higher than those in the first cycles despite the 2-day interval between the first and second cycles (Fig. 2). The intervals of 2 days or more between cycles were considered to be enough for the plasma concentrations of 5-FU to be reset to zero at the beginning of the drug infusion in all cycles, although this was not checked in the present study. Interestingly, the variation of plasma drug concentrations between 5 AM and 5 PM in the second cycle was smaller than that in the first cycle.

Third, analyzed according to course, these phenomena as described above were observed in both the first and second courses. Furthermore, plasma concentrations in the first cycle of the second course were lower than those in the second cycle of the first course, and similar with those in the first cycle of the first course. In short, reproducibility was demonstrated in e first and second courses.

These results suggested that the circadian variation of the plasma concentrations of 5-FU might converge gradually with the elevation of plasma levels. Interestingly, a 2-day interval could be insufficient to recover it, but a 3-week interval could be sufficient. This is possibly a result of the tolerance and recovery system of metabolic enzymes after the long exposure to 5-FU. The elimination of 5-FU takes place primarily by a saturable metabolic process and possibly achieves a saturated state with rapid bolus injection of a high dosage of 5-FU.[33] However, its metabolism may be unlikely to saturate at the concentration of at least 0.2 μg/mL in plasma in the case of PVI, and the theory of drug saturation cannot by itself explain our findings.

The pharmacokinetics of 5-FU have been investigated from the viewpoint of circadian rhythm in catabolic activities of dihydropyrimidine dehydrogenase (DPD), which is an initial and rate-limiting enzyme in the degradation of 5-FU.[18,35,36] To date, dose-modified or chronomodulated chemotherapy has been used in consideration of circadian rhythms of DPD activity, drug concentrations and cell cycles.[27,37,38] However, it is very complicated to take these methods into clinical practice because of the required frequent samplings and variable circadian rhythms of 5-FU concentrations.[19,29] Furthermore, the relationship between 5-FU concentrations and DPD activities was not elucidated despite frequent samplings.[30,39] In the present study, fluctuating patterns of plasma concentrations were highlighted because even frequent samplings appear to have a limit in detailed explanation of pharmacokinetics of 5-FU. Here, in an attempt to determine a new and simple possible candidate that influences the circadian variation of drug concentrations, we focused on *CLOCK* genetic polymorphism. The *CLOCK* gene has a pivotal role in the generation and control of circadian rhythms.[22] The significance of *CLOCK T3111C* polymorphism has been reported in the psychiatric field,[23,26] but no previous reports were available about its impact on the pharmacokinetics of 5-FU. Our administration schedule of 5-FU is suitable to investigate its possible impact because the reproducibility of the same fluctuating patterns of plasma concentrations was confirmed. To keep similar surroundings, patients hospitalized at the same hospital with the same disease were treated with the same regimen. The result did not show that *CLOCK T3111C* polymorphism had a statistically significant impact on CV values in the first and second cycles, although this SNP tended to give a lower fluctuation of the concentration (Fig. 3). This result was not different when CV values were analyzed according to phase, cycle, and course (data not shown). Unfortunately, blood sampling at other times could not be allowed in consideration of each patient's burden, and it remains unclear whether this result may be influenced by the progression of DPD activity, the clinical background of our study population, the shift of daily active–inactive phase, or other factors.

In conclusion, (1) pharmacokinetics of 5-FU may exhibit substantially higher plasma concentrations in the evening than in the morning, (2) plasma concentrations of 5-FU significantly increased at the second cycle of both first and second courses of chemotherapy despite the interval of 2 days between the first and second cycles and despite the very short half-life of 5-FU (4–25 minutes), probably because of the methods of administration, and (3) the variability in plasma concentrations of 5-FU was not statistically significant between each *CLOCK T3111C* genotype in this Japanese population. In 5-FU-based chemotherapy, the administration schedule should be made in consideration of these phenomena. Further studies are needed to clarify the mechanism of these phenomena and to determine an easy-to-assess marker of its circadian variation, yielding a clinical impact on treatment outcome for use in individualizing delivery of 5-FU.

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胃癌の腫瘍マーカーとその活用

辻　晃仁*

要旨

腫瘍マーカーは簡便な臨床検査である。しかし，現在の腫瘍マーカーは，胃癌に特異的なものであるとはいえず，かつ早期癌における陽性率は低いのが現状である。このため，CA 19-9，CA 50，SPan-1，CA 72-4，STn，NCC-ST-439，SLX，CEAなど各種の腫瘍マーカーを，臨床診断にあわせて組み合わせ，使い分けている。

胃癌ハイリスク患者のスクリーニングには，ペプシノーゲン・アイソザイムI/II（PG I/II）比やヘリコバクター・ピロリ血中（HP）抗体が用いられている。

また，胃癌の腫瘍マーカー陽性率は，一般的にはstage III以上のもので高いとされるが，高齢者，気管支炎，慢性膵炎，慢性肝炎，糖尿病などでは偽陽性を示すことも多く注意が必要である。

AFP産生胃癌は転移しやすく，予後不良であることが多い。

stage I，IIの患者においては，術前血清CEA高値の患者で再発症例が多く，潜在性の転移の可能性が高いとされる。特に，高度上昇例ではリンパ節転移や他臓器（肝や肺など）の転移が疑われる。

術前CA 19-9値は深達度，リンパ節転移，遠隔転移に次ぐ重要な予後因子とされる。

腹膜播種がある際のCA 125の陽性率は高い。

化学療法時，血中腫瘍マーカー値の50%以下への低下は，化学療法の効果と生命予後の延長効果を予測する指標になるとされる。

今後，さらに特異性の高い，また早期より検出可能な胃癌の腫瘍マーカーの開発が期待される。

診断における腫瘍マーカーの意義

胃癌における特異的な腫瘍マーカーは現時点では確立されていない。このため存在診断としての意義よりも，術前の進行度，転移の有無や術後の再発follow-upとしての有用性が高いと考えられている。臨床的にはCEA，CA 19-9などの腫瘍マーカーを，臨床診断にあわせて組み合わせ，使い分けているのが現状である。

1．胃癌ハイリスク患者のスクリーニング

腫瘍マーカーの存在診断の意義は決して高くないが，胃癌のハイリスク群のスクリーニングに以下のものが用いられている。

1）ペプシノーゲン・アイソザイムI/II（PG I/II）比

胃癌のハイリスク群とされる慢性萎縮性胃炎と腸上皮化生をスクリーニングする方法である[1]。

一部の住民健診，職場健診で従来の間接二重造影法に変えてスクリーニングに用いる試みも始まっている。

2）ヘリコバクター・ピロリ血中（HP）抗体

HP抗体は，WHOにより胃癌の危険因子の一つとされ，ハイリスク患者のスクリーニングに期待されている。

HP抗体IgG検査実施診断料は保険適用で70

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点となる。類似検査である便中HP抗原は160点，尿素呼気試験は70点である。

ただヘリコバクター・ピロリ菌の除菌療法が，胃癌予防に有用であるかはまだ証明されていない。

2．胃癌診断の補助となるマーカー

・シアリルLe^{a}グループ：CA 19-9，CA 50，SPan-1
・シアリルTn抗原グループ：CA 72-4，STn
・シアリルLe^{x}抗原グループ：NCC-ST-439，SLX
・癌胎児性抗原：carcinoembryonic antigen (CEA)

1）胃癌の腫瘍マーカー

胃癌の腫瘍マーカーとしては従来CEA[2]，CA 19-9などが広く測定されてきた。

胃癌の腫瘍マーカーは，一般に分化型胃癌に対し低分化型胃癌の陽性率は低いとされる[3]。

ただ胃癌全体では，CEAよりもCA 19-9，CA 50，SPan-1などのシアリルルイスAグループの糖鎖抗原や，CA 72-4，STn，CA 546などのシアリルTn抗原グループの陽性率が高く，予後との相関も高いとされている。

また，CA 72-4などのシアリルTn抗原と，NCC-ST-439などのシアリルLe^{x}抗原グループは進行癌において陽性率が高く，偽陽性率も低い。胃癌の治療効果のマーカーとしても期待される。

2）臨床における腫瘍マーカーのとらえ方

一般的に胃癌の腫瘍マーカー陽性率は，stage III以上のもので高い[4]（図1）。

CEAが非常に高いもの（30 ng/ml以上）などの場合は，肝転移やリンパ節転移を疑うことが多い。

偽陽性は高齢者，気管支炎，慢性膵炎，慢性肝炎，糖尿病などに多く，10 ng/ml以下のものが多い傾向にある。

現在の保険診療上，癌を疑う場合の腫瘍マーカー精密測定の検体検査実施料は，1検体では検査の種類によって異なり130～310点（多くは200点以下）であるが，2項目では270点，3項目では340点，4項目以上では460点のいわゆる「まるめ」となる。また，癌の診断が付いているものについては，管理料として1項目360点，2項目以上では400点（初回月は＋150点加算）が適応される（表1）。

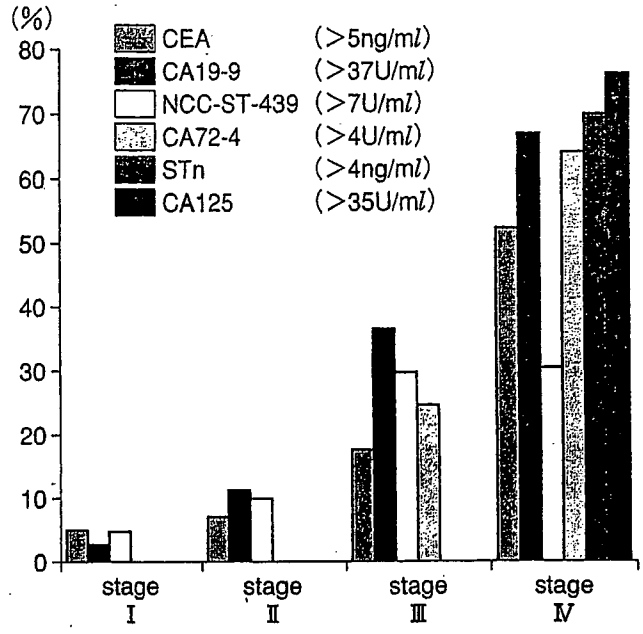

図1　胃癌における腫瘍マーカーの陽性率
（文献[4]より引用し改変）

3．胃癌のサブタイプ鑑別を行う腫瘍マーカー

胃癌の陽性率は高くないが，特殊なタイプの胃癌であることを示す腫瘍マーカーにAFPとhCGがある。

1）AFP

AFP産生胃癌は転移しやすく（特に肝転移が多い），AFP高値（20 ng/ml以上）の症例は予後不良であることが知られている（図2）[5]。

AFPの検体検査実施料は1項目40点，2項目以上では75点である（癌の診断が付いているものについては，管理料として一律220点が適応される）。

2）hCG

hCG産生胃癌は，絨毛癌と似た性質を持ち，胃の絨毛癌と分類されることもある[6]。化学療法の反応性も比較的良い。

hCGの検体検査実施料は1検体では180点，2項目270点，3項目340点，4項目以上では460点である。

表1　検体検査実施料と悪性腫瘍特異物質治療管理料

検査項目名		悪性腫瘍であることが強く疑われる患者		悪性腫瘍であるとすでに診断が確定した患者
		検体検査実施料		悪性腫瘍特異物質治療管理料
一般測定	αフェトプロテイン（AFP）	40点	2項目以上 75点	220点
	免疫抑制酸性蛋白（IAP）	60点		
	尿中BTA*1	(70点)		
精密測定	癌胎児性抗原（CEA）精密測定 αフェトプロテイン（AFP）精密測定 組織ポリペプチド（TPA）精密測定	130点		1項目　360点 2項目以上　400点 初回月加算　150点 当該初回月の前月に腫瘍マーカー検査実施料の所定点数を算定している場合は，当該初回月加算は算定できない
	扁平上皮癌関連抗原（SCC抗原）精密測定	140点		
	DUPAN-2精密測定 NCC-ST-439精密測定 CA 15-3精密測定	150点		
	エラスターゼ1精密測定 前立腺酸性フォスファターゼ（PAP）精密測定 前立腺特異抗原（PSA）精密測定	160点		
	塩基性フェトプロテイン（BFP）精密測定 CA 19-9精密測定 CA 72-4精密測定 CA 50精密測定 SPan-1抗原精密測定 シアリルTn抗原（STN）精密測定 尿中NMP 22精密測定*2 神経特異エノラーゼ（NSE）精密測定 PIVKA-II精密測定	170点		
	SP1精密測定 シアリルLe^x-i抗原（SLX）精密測定 CA 125精密測定	180点	2項目　270点 3項目　340点 4項目以上　460点	
	尿中遊離型フコース（UFC） シアリルLe^x（CSLEX）精密測定 フリーPSA/トータルPSA比精密測定	190点		
	BCA 225精密測定	200点		
	CA 602精密測定 CA 130精密測定 サイトケラチン19フラグメント（シフラ）精密測定 ガストリン放出ペプチド前駆体（ProGRP）精密測定	210点		
	AFPのレクチン反応性による分画比（AFP-L 3%） CA 54/61精密測定 癌関連ガラクトース転移酵素（GAT）精密測定	220点		
	膵癌胎児性抗原（POA）精密測定 γ-セミノプロテイン（γ-Sm）精密測定	230点		
	尿中ヒト絨毛性ゴナドトロピンβ分画コア定量（HCG-βコア定量）精密測定	240点		
	乳頭分泌液中CEA精密測定 乳頭分泌液中HER 2タンパク測定 血清中HER 2タンパク測定	310点		
	I型コラーゲン-C-テロペプチド（ICTP）精密測定 I型コラーゲン架橋N-テロペプチド（NTx）精密測定 尿中デオキシピリジノリン精密測定 I型プロコラーゲン-C-プロペプチド（PICP）精密測定			

[注]
＊1：「悪性腫瘍特異物質治療管理料」として，のみ算定。
＊2：「検体検査実施料」として，のみ算定。

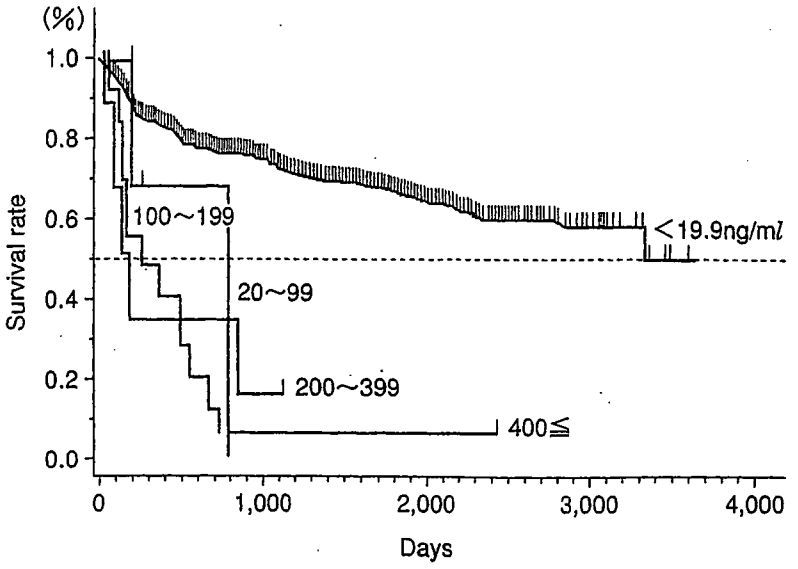

図2　術後 AFP 値と胃癌患者の予後

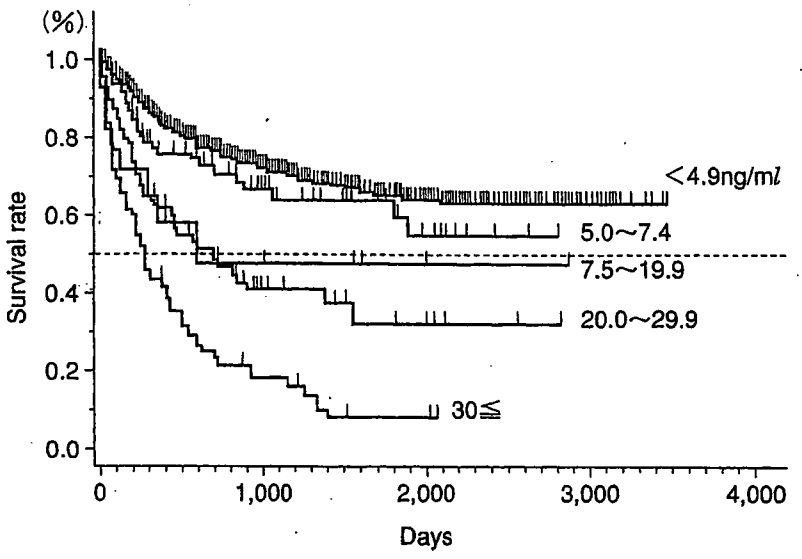

図3　術前 CEA 値と胃癌患者の予後

経過観察時の腫瘍マーカーの意義

1．経過観察のモニターおよび予後因子としての腫瘍マーカー

血清腫瘍マーカーが基準値をわずかに超えるような場合は，その腫瘍マーカーが実際に陽性であるかの判断は困難である。ただ手術，化学療法開始後に血中レベルの低下があったり，経過観察時に経時的に増加したりする場合には経過観察時のモニターとなり得る。

一般的には画像診断で癌の再発が発見されるよりも，1～6カ月前（リードタイムと呼ばれる）より血清腫瘍マーカー値の上昇が観察されることが多い。

1）CEA

stage Ⅰ，Ⅱの患者のうち，術前血清 CEA が高値の患者には再発症例が多く，潜在性の転移の可能性が高いとされる（図3）[7]。

また，20.0 ng/ml 以上の高度上昇例では，リンパ節転移や他臓器（肝や肺など）の転移が疑われる。

CEA 値の上昇で，比較的緩徐な上昇の際には局所再発が，急速な増加を示す際には血行性転移が

多いとされる[8]。

2）CA 19-9

胃癌全体でのCA 19-9の陽性率は25～30%である。

ただし胆管炎や胆道閉塞，糖尿病，間質性肺炎などで高値を示すことが多い（100 U/m*l* を超えることは少ない）。

術前CA 19-9値は深達度，リンパ節転移，遠隔転移に次ぐ重要な予後因子とされる[9]。

3）CA 125

腫瘍が漿膜に達した場合と，腹膜播種のある場合に陽性となることが多い。

腹膜播種がある際の陽性率は，他の腫瘍マーカーが10%以下と低率であるのに対して，40%以上の高い感度である。

ただ他の腫瘍マーカーと異なり，胃癌細胞からはほとんど産生されず，腹膜播種に伴う腹膜炎により上昇しているものと推測されているが，実際の臨床の場では，胃癌に腹膜炎を伴う症例の多くは癌性腹膜炎であり，腹膜播種は画像診断での指摘が困難なものも多く経験されるため，CA 125の有用性は高い。

4）AFP

肝転移におけるAFPの陽性率は10%前後で決して高値ではないが，AFP陽性の胃癌患者の肝転移率は70%を超えるとの報告もある[10]。

5）FDP

stage IV症例にのみ異常値がみられ，高度の脈管侵襲による血管内凝固異常の存在と予後不良を示唆する。

6）IAP（免疫抑制酸性蛋白）

IAPは宿主の免疫能を反映するマーカーで，厳密には腫瘍マーカーとは異なるが予後の予測に有用とされる[11]。

7）肝転移

胃癌肝転移症例における腫瘍マーカーの陽性率はCEA：38～65%，CA 19-9：35～60%，AFP：8～15%，CA 125：15%である（表2）。

8）腹膜播種

胃癌腹膜播種症例における腫瘍マーカーの陽性率はCEA：8～9%，CA 19-9：7～9%，AFP：0～1%，CA 125：43～52%である[12]（表2）。

表2　胃癌肝転移，腹膜播種と腫瘍マーカー

	肝転移	腹膜播種
CEA	38～65%	8～9%
CA 19-9	35～60%	7～9%
AFP	8～15%	0～1%
CA 125	15%	43～52%

（文献[12]より引用し改変）

表3　胃癌再発とCEA，CA 19-9

	術前陽性率	再発時陽性率
CEA	28.3%	65.8%
CA 19-9	29.2%	55.0%
Either/both	45.0%	85.0%

9）リンパ節

リンパ節転移の予測にはCEA，CA 19-9が有用である。どちらかもしくは双方が陽性の場合の特異度は90%以上とされる[13]。

10）再発時

CEA，CA 19-9ともに陽性率が高まるとされている[14]（表3）。

11）化学療法時の治療経過のマーカー

腫瘍マーカー値の50%以下への低下は，化学療法の効果と生命予後の延長効果を予測する指標になるとされる[15]。

2．腹水などの検体中の腫瘍マーカー

腹水・腹腔洗浄液中CEA

腹膜播種の指標として一部施設で，細胞診などと併用で用いられている。

おわりに

腫瘍マーカーは簡便な臨床検査である。しかし，現在の腫瘍マーカーは，胃癌に特異的な腫瘍マーカーであるとはいえず，かつ早期癌における陽性率は低い。このため，現時点での腫瘍マーカーの意義は，以下のようなものであると考えられる。

①特異的な腫瘍マーカーは少ないが，各種腫瘍マーカーを組み合わせることにより術後の再発，転移の発見に有用な情報となり得る。

②腫瘍マーカー値の経時的な変化が，手術，化学療法，放射線療法などの各種治療の効果判定の

良い指標となることが多い。

③画像診断が困難な腹膜播種に対するCA 125や，画像診断可能となる時期以前のリンパ節転移に対するCEA, CA 19-9などの症例において有効な診断情報となる。

今後，特異性の高い，また早期より検出可能な胃癌の腫瘍マーカーの開発が期待される。

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ORIGINAL ARTICLE

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A phase I study of doxifluridine combined with weekly paclitaxel for metastatic gastric cancer

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Abstract *Purpose*: Based on the synergistic effect in preclinical studies, a phase I clinical trial for the combination of paclitaxel and doxifluridine (an intermetabolite of capecitabine) was performed to determine the recommended dose for the treatment of patients with metastatic gastric cancer. *Methods*: The dose of paclitaxel was increased from 60 mg/m^2 at level 1 to 90 mg/m^2 at level 5. It was administered as a 1-h infusion on days 1 and 8. The dose of doxifluridine was fixed at 600 mg/m^2 per day up to level 3, and escalated to 800 mg/m^2 per day at levels 4 and 5. It was administered orally for 2 weeks. The treatment was repeated every 3 weeks. *Results*: A total of 28 patients were enrolled. No dose-limiting toxicity (DLT) was observed at levels 1 and 2 (paclitaxel 70 mg/m^2). A DLT of grade 4 neutropenia lasting for more than 4 days was observed in one patient at level 3 (paclitaxel 80 mg/m^2). In addition, the first five of six patients in this group experienced grade 3 neutropenia during the first treatment cycle. A further six patients were added in order to confirm the safety of this dosage level, and no more DLTs except for grade 3 nausea in one patient were observed in the second cohort. No DLT was seen in three patients at level 4 (paclitaxel 80 mg/m^2). DLTs (grade 3 neuropathy in one patient and a treatment delay of the second cycle for more than 1 week due to grade 3 neutropenia in another) were observed in two out of six patients at level 5 (paclitaxel 90 mg/m^2), and this dose level was determined as the maximum tolerated dose. The tumor response rate was 42% (95% confidence interval 20–67%) in 19 patients with measurable lesions. *Conclusions*: The recommended dose was determined as 80 mg/m^2 of paclitaxel (days 1 and 8) and 800 mg/m^2 of doxifluridine (days 1–14) every 3 weeks. The results of this phase I study are encouraging and a phase II trial is thus warranted.

Keywords Doxifluridine · Thymidine phosphorylase · Taxane · Gastric cancer · Clinical trial

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Introduction

The incidence of gastric carcinoma is still high in Asia and it remains one of the leading causes of death [13, 28]. The prognosis for patients with unresectable or metastatic gastric carcinoma is poor, but chemotherapy confers a benefit when compared with best supportive care alone [9, 23]. In the past over 20 years, several anticancer drugs such as 5-fluorouracil (5-FU), cisplatin, methotrexate, doxorubicin, epirubicin, mitomycin, and etoposide, have been studied either alone or in combination as treatments for this disease. However, no new combination has yet emerged that is superior to 5-FU alone or to 5-FU plus cisplatin in terms of overall survival [13, 22, 31]. There is a pressing need for the evaluation of new agents such as the oral fluoropyrimidines and taxanes.

Paclitaxel promotes microtubule assembly and then exhibits its antitumor effect by arresting the cell cycle in the G_2/M phase. This mechanism of action is different from conventional anticancer drugs, and it has therefore been suggested that combination therapy with other anticancer drugs may be clinically effective [17]. The efficacy of paclitaxel has previously been confirmed clinically in various tumors including gastric cancer [1, 5, 10, 18, 19, 21, 33]. Furthermore, some promising regimens of paclitaxel combined with 5-FU/leucovorin/cisplatin, or with 5-FU/cisplatin have been reported in advanced gastric cancer [11, 14].

Preclinical studies have shown that paclitaxel induces thymidine phosphorylase (dThdPase) specifically in various human tumor tissues [26]. The oral fluoropyrimidine capecitabine and its intermetabolite doxifluridine are prodrugs that are converted to 5-FU by dThdPase in tumor tissues [6, 12]. A synergistic effect on inhibition of tumor growth has been reported when these agents are combined with paclitaxel [26]. Modest activity of capecitabine and doxifluridine has been reported in the treatment of advanced gastric cancer [7, 15, 20, 32]. Doxifluridine was approved for use in the treatment of advanced gastric cancer in 1987 in Japan, but capecitabine is still under investigation for this disease.

Thus, we conducted a phase I clinical trial in order to study the feasibility of paclitaxel/doxifluridine combined therapy. The tumor response was also investigated.

Patients and methods

Patients

All patients had to fulfill the following eligibility criteria: (1) histological confirmation of gastric adenocarcinoma; (2) inoperable metastatic disease or recurrent metastatic disease after surgery; (3) measurable or evaluable lesions; (4) aged from 20 to 75 years; (5) performance status (PS) ≤ 2 on the Eastern Cooperative Oncology Group (ECOG) scale; (6) a maximum of one prior chemotherapy other than paclitaxel or doxifluridine for advanced disease (prior chemotherapy for advanced disease must have been completed at least 4 weeks prior to enrollment); (7) adequate bone marrow function (absolute granulocyte count $\geq 1500/mm^3$ and platelet count $\geq 100{,}000/mm^3$; (8) adequate liver function (serum bilirubin < 1.5 mg/dl and serum transaminase < 100 U/l); (9) adequate renal function (serum creatinine < 1.2 mg/dl); (10) no other severe medical conditions; (11) no other active malignancies; (12) no pregnant or lactating patients; (13) no peripheral neuropathy; and (14) provision of written informed consent.

This study was approved by the Institutional Review Board of the National Shikoku Cancer Center.

Dose-limiting toxicity and maximum tolerated dose

Dose-limiting toxicities (DLTs) were determined during the first treatment cycle. The definitions of DLTs were as follows: (1) grade 4 neutropenia lasting for at least 4 days, or grade 3 or 4 neutropenia with fever, (2) grade 4 thrombocytopenia, (3) grade 3 non-hematological toxicity, and (4) treatment delay of more than 2 weeks following the last administration of doxifluridine. The maximum tolerated dose (MTD) was defined as the dose level at which two of the three to six treated patients experienced DLT, and the recommended dose (RD) was determined at one level below.

Baseline evaluation included a complete medical history, physical examination, complete blood cell count, serum chemistry, urinary analysis, ECG, gastroscopy, gastrography, abdominal CT scan, and chest radiography. Blood, chemistry, urinary analyses, and subjective/objective symptoms for toxicity were monitored on a weekly basis during the treatment. Blood cell counts were determined at least every 2 days if hematological toxicities of grade 3 or more were seen in the first treatment cycle. When patients received the subsequent treatment cycle, they had to fulfill the previous eligibility criteria (7), (8), and (9), and their non-hematological toxicities had to recover to grade 1.

Toxicities were evaluated according to the National Cancer Institute common toxicity criteria (version 2.0).

Dosage and administration

The previous reports of phase I clinical trials studying the weekly administration of paclitaxel as a single agent in breast and ovarian cancer revealed that the RD was 80–100 mg/m^2 [16, 27]. We set the starting dose of paclitaxel (Taxol; Bristol-Myers Squibb Company, Tokyo, Japan) at 60 mg/m^2 and the dose was escalated by 10 mg/m^2 for each dose level up to dose level 3. Paclitaxel dissolved in 500 ml of an isotonic sodium chloride solution was administered on days 1 and 8 as an intravenous (i.v.) drip injection over 60 min following the short premedication (dexamethasone sodium phosphate 20 mg i.v. drip, diphenhydramine hydrochloride 50 mg orally, and ranitidine hydrochloride 50 mg i.v. 30 min before paclitaxel administration). Because 600–800 mg/m^2 per day of doxifluridine (Fulturon; Chugai Pharmaceutical Company, Tokyo, Japan) was considered the dose for patients with gastric cancer and this dose had been approved as the single-agent RD in Japan [20, 33], we fixed doxifluridine at the dose of 600 mg/m^2 per day and administered it orally at regular intervals four times a day (after each meal and before sleep) for 14 days. If the MTD did not reach level 3, the dose of each drug in the subsequent level was escalated in tandem by 10 mg/m^2 of paclitaxel and by 200 mg/m^2 of doxifluridine as shown in Table 1.

This treatment was repeated every 3 weeks (one cycle each) until disease progression or unacceptable toxicity was seen. The first cycle of the treatment was performed in the in-patient setting in our center. If the patient experienced DLT followed by no disease progression, the subsequent cycle was started at the next lower level after complete recovery from the toxic effects of the previous cycle.

Tumor response

Tumor response was evaluated every 6 weeks by means of CT scan. Measurable lesions were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) [30].

Table 1 Dose level, number of patients enrolled, and DLT

Level	Paclitaxel (mg/m^2)	Doxifluridine (mg/m^2)	No. of patients	DLT
1	60	600	4	None
2	70	600	3	None
3	80	600	12	One grade 4 neutropenia lasting more than 4 days; one grade 3 nausea
4	80	800	3	None
5	90	800	6	One grade 3 neuropathy; one treatment delay due to neutropenia

Results

A total of 28 patients were enrolled, with 4 patients dosed at level 1, 3 at level 2, 12 at level 3, 3 at level 4, and 6 at level 5 from September 2001 to January 2003 (Table 1). Because one patient dosed at level 1 developed a grade 1 hypersensitivity reaction during the first treatment cycle and refused further treatment, a replacement patient was added to this dosage group. The patient characteristics are shown in Table 2. Of the 28 patients, 21 exhibited a good PS (0 or 1), and 22 had had a prior chemotherapy. The most frequent prior chemotherapy was 5-FU (17 patients). Nine patients had differentiated histological gastric adenocarcinoma, and the remainder had the undifferentiated type. The major metastatic sites were peritoneum, lymph nodes and liver.

The adverse events in the first cycle are summarized in Table 3. The most frequently observed toxicity was neutropenia. DLTs were not observed at levels 1 and 2, but a DLT (grade 4 neutropenia which continued for more than 4 days) was observed in the second patient at level 3. Then three patients were added to this dosage group. No DLT was observed in these additional patients. However, grade 3 neutropenia was observed in five patients (83%) in the first treatment cycle at this dose level. In addition, a 1-week postponement of the second cycle was needed due to the neutropenia in one patient and grade 4 neutropenia developed in another patient in the second cycle. Therefore, an additional six patients were enrolled in order to confirm the safety of this dose level. No DLT except for grade 3 nausea in one patient was observed in this second cohort, and we moved to the next dosage level. At level 4, grade 3 neutropenia was observed in two of three patients. However, no DLT was seen in this cohort. DLT (more than a 1-week treatment delay due to grade 3 neutropenia) was observed in the third patient at level 5. Three patients were added to this level. DLT (grade 3 peripheral neuropathy) was observed in the sixth patient. Grade 2 neuropathy appeared following the first administration of paclitaxel on day 1 and increased to grade 3 immediately after the second administration on day 8. The treatment was continued up to three cycles at the next lower dosage level, although grade 1 or 2 peripheral neuropathy developed during every cycle. From these results, level 5 was determined as the MTD and level 4 (paclitaxel 80 mg/m^2, doxifluridine 800 mg/m^2) was set as the RD. The lowest neutrophil counts in the first cycle at each dosage level are shown in Table 4. The medians of the lowest absolute neutrophil counts were graded as grade 3 neutropenia in levels 3, 4 and 5. Their values were apparently lower than those in levels 1 and 2. The period of recovery to grade 1 was around a week in levels 3, 4, and 5. It was also longer than that in levels 1 and 2.

The main toxicity of this combined therapy was myelotoxicity, neutropenia in particular. Grade 3 or 4 neutropenia was observed in 0 of 12 cycles (0%) at level 1, 1 of 20 cycles (5%) at level 2, 14 of 76 cycles (18%) at level 3, 3 of 13 cycles (23%) at level 4, and 3 of 15 cycles (20%) at level 5. Non-hematological toxicities of greater than grade 3 were observed in four patients during all treatment cycles. Two of these were the DLT. One of the remaining two patients showed grade 3 diarrhea in the fourth cycle at level 4, and the other patient showed grade 3 peripheral neuropathy after five cycles at level 5. A total of seven patients needed dose reduction during all treatment cycles. Four patients with DLT (Table 1) and two patients with grade 3 diarrhea and grade 3 peripheral neuropathy, respectively, were included. The other was the patient who showed grade 4 neutropenia in the second cycle at level 3. Peripheral neuropathy of grade 1 or 2 occurred in 2 of 12 patients at level 3, 1 of 3 patients at level 4, and 3 of 6 patients at level 5. It tended to be more severe following repeated administration of paclitaxel and seemed cumulative. Hand-foot syndrome

Table 2 Characteristics of patients

Age (years)	
Median	63
Range	44–75
Sex	
Male/female	16/12
Performance status (ECOG)	
0/1/2	10/11/7
Prior therapy	
Gastrectomy	20
Chemotherapy (5-FU)	22 (17)
Histological type	
Differentiated	9
Undifferentiated	19
Sites of metastasis	
Liver	6
Abdominal lymph nodes	17
Lung	5
Peritoneum	19
Spleen	2

Table 3 Adverse reactions during the first treatment cycle

Toxicity	Level 1 ($n=4$)		Level 2 ($n=3$)		Level 3 ($n=6$)				Level 4 ($n=3$)		Level 5 ($n=6$)	
	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4
Hematological												
Leukopenia	2	0	2	1	5	1	1	2	2	0	5	0
Neutropenia	3	0	2	1	1	5[a]	3	2	0	3	3	2[a]
Lymphocytopenia	4	0	1	1	1	0	1	1	2	0	6	0
Anemia	3	1	3	0	4	1	5	0	0	2	4	1
Thrombocytopenia	2	0	0	0	0	0	2	0	0	0	1	0
Non-hematological												
Hypersensitivity reaction	1	0	0	0	0	0	0	0	0	0	0	0
Infection	0	0	0	0	0	0	0	0	0	0	0	0
Fatigue	2	0	0	0	1	0	1	0	3	0	0	0
Nausea	2	0	0	0	0	0	1	1[a]	0	0	2	0
Vomiting	0	0	0	0	0	0	1	0	0	0	0	0
Anorexia	1	0	0	0	1	0	0	0	2	0	1	0
Diarrhea	0	0	0	0	2	0	1	0	2	0	0	0
Stomatitis	1	0	0	0	0	0	0	0	0	0	1	0
Alopecia	1	–	0	–	3	–	2	–	2	–	5	–
Edema	0	0	0	0	0	0	0	0	0	0	0	0
Hand-foot syndrome	0	0	0	0	0	0	0	0	0	0	0	0
Rash	1	0	0	0	0	0	1	0	0	0	0	0
Palpitations	0	0	0	0	0	0	1	0	1	0	0	0
Peripheral neuropathy	0	0	0	0	0	0	0	0	1	0	0	1[a]

[a]DLT was observed in one patient

Table 4 Lowest absolute neutrophil count (LNC) during the first treatment cycle

Level	LNC (per mm^3)		Time to LNC (days)		Recovery from LNC (days)	
	Median	Range	Median	Range	Median	Range
1	1860	1287–1884	14	12–22	2	2–6
2	1700	558–1768	14	4–17	3	2–10
3	904	200–1672	14	6–16	8	4–15
4	867	738–996	14	14	8	7–8
5	902	801–1824	14	13–17	6	6–21

was not observed in any patient during any treatment cycle. There was no treatment-related death.

The median numbers of administration cycles were 2 (range 1–8) for level 1, 6 (range 3–11) for level 2, 6 (range 2–13) for level 3, 4 (range 3–6) for level 4, and 3 (range 2–5) cycles for level 5.

An objective tumor response was not observed in the patients at dosage levels 1 and 4. Three patients showed a partial response (PR) at level 2. Two patients showed a complete response (CR) and one patient showed a PR at level 3. Two patients showed a PR at level 5. The overall response rate in all 19 patients with measurable lesions was 42% (95% confidence interval 20–67%). The response rate in pretreated patients was 43% (6/14) and that in chemonaive patients was 40% (2/5). The ascites disappeared in two of three patients without measurable lesions at level 3.

Discussion

Based on the results of phase I and II clinical trials of paclitaxel, the RD was set at 210 mg/m^2 over a 3-week dosing schedule in Japan, and a relatively high tumor response rate of 23% has been reported for advanced gastric cancer [29, 33]. In addition, paclitaxel yielded the same response rate in the second-line setting (23%) as in the first line setting (24%), and non-cross resistance with other anticancer drugs was suggested. In terms of the toxicity, leukopenia and neutropenia of higher than grade 3 were observed in 28% and 58% of patients, respectively. These results are supported by other studies [1, 5]. In recent years, the concept of dose-dense therapy whereby the interval between administrations is shortened to reduce the time for regrowth of neoplastic cells has been proposed [8]. Several clinical studies involving weekly dose-dense therapy of paclitaxel have been performed in lung, breast, and ovarian cancer [16, 24, 27]. Following a phase I clinical trial in 60 patients with advanced cancer who had been treated previously with systemic chemotherapy other than taxanes, the RD was 80 mg/m^2 of paclitaxel weekly, and grade 3 or higher toxicities were rarely observed [16]. In addition, a recent randomized trial comparing two administration methods of paclitaxel with the same dose intensity (conventional 3-week regimen of 200 mg/m^2 and weekly regimen of 67 mg/m^2) in patients with recurrent ovarian cancer revealed equal response rates and overall survival, and reduced toxicities with the weekly schedule [24]. Thus, weekly dosing of paclitaxel has been confirmed to have equal efficacy and lower toxicity than the conventional dosing regimen.

Capecitabine is still under investigation for advanced gastric cancer in Japan. Instead, the immediate precursor to capecitabine, doxifluridine, has been approved for the treatment of advanced gastric cancer. In preclinical evaluations, the therapeutic index of doxifluridine has been shown to be much more than that of 5-FU [4]. In clinical studies, high oral bioavailability of doxifluridine has been noted, and it has shown prominent antitumor activity in patients with breast, colorectal, and gastric cancers [2, 7, 20]. The DLT of doxifluridine is diarrhea. Recently, it has been reported that several anticancer drugs, including paclitaxel, upregulate the expression of dThdPase specifically in tumor tissues and that paclitaxel in combination with doxifluridine shows the synergistic activity in several human cancer xenograft models [26]. Furthermore, the major toxicities of paclitaxel and doxifluridine do not overlap. Therefore, we adopted a weekly dosing regimen of paclitaxel in combination with doxifluridine.

Neutropenia was the most frequently observed toxicity with this combination therapy, and was dose-limiting. However, no neutropenic fever was seen in this study. At dose level 3, one patient experienced a DLT (grade 4 neutropenia for more than 4 days), and five of six patients, including the patient with DLT, exhibited grade 3 or more neutropenia in the first treatment cycle. Based on these results, the investigators and the independent efficacy and safety committee considered it appropriate to stop dose loading at level 3. However, the MTD was not achieved at this level. Then six patients were added to confirm the safety of this dose level. No DLT except for a patient with grade 3 nausea was observed in this additional cohort. Thus, dose escalation was reopened and no grade 4 neutropenia was observed at level 4. Non-hematological toxicity was generally mild. Peripheral sensory neuropathy, one of DLTs of paclitaxel, was well tolerated up to level 4, and diarrhea, a DLT of doxifluridine, was not severe in the initial few cycles at all levels. At level 4, there was only one patient who needed dose reduction due to diarrhea after four treatment cycles. The median numbers of cycles administered were 6 (range 2–13) at level 3 and 4 (range 3–6) at level 4. The main reason for stopping the treatment was disease progression at levels 3 and 4. From

these results, the dosage schedule at level 3 or 4 seems to be highly feasible.

Conventional 3-h infusion of paclitaxel with a 3-week interval combined with infusional 5-FU and cisplatin was studied in Korean group in a phase II trial in advanced gastric cancer [14]. A high response rate of 51% and good tolerability was reported in this study. Recently, it has been reported from Germany that weekly administration of paclitaxel with a combination of 5-FU/folinic acid and cisplatin showed a reduced incidence of hematological toxicity, particularly leukopenia, and other toxicities, apart from a slightly higher incidence of peripheral neuropathy, were also comparable between the weekly regimen and the conventional regimen [11]. The response rate (50%) in this German phase II study in advanced gastric cancer was well maintained with the weekly regimen.

Active oral fluoropyrimidines, such as capecitabine, S-1, and uracil/ftorafur (UFT) plus leucovorin have recently been developed [3, 15, 25]. Based on promising reports, trials are being urgently undertaken in many countries to determine whether 5-FU combined with various agents could be replaced by these new oral fluoropyrimidines. Although most patients in our study had received prior chemotherapy, this doxifluridine and paclitaxel combined therapy yielded a high response rate of 42% (95% confidence interval 20–67%). In addition, elimination of ascites was observed in two of three patients. The efficacy of this combination therapy would also be expected in patients with peritoneal dissemination that is frequently seen in advanced gastric cancer. These results encouraged us to move to further trials.

In conclusion, we performed a phase I clinical trial using a combination of paclitaxel and doxifluridine, and determined the RD as 80 mg/m^2 of paclitaxel on days 1 and 8, and 800 mg/m^2 per day of doxifluridine for 2 weeks in a 3-week treatment schedule. The results of our present study are promising and a phase II clinical trial of this combination therapy is planned in which the safety of the RD will be investigated carefully in the first six or more patients.

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ORIGINAL ARTICLE

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In vitro schedule-dependent interaction between paclitaxel and oxaliplatin in human cancer cell lines

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Abstract *Purpose*: In order to define the most effective administration schedule of the combination of paclitaxel and oxaliplatin, we investigated the in vitro interaction between these drugs in a panel of three human cancer cell lines (AZ-521 gastric adenocarcinoma cell line, HST-1 tongue squamous carcinoma cell line, and KSE-1 esophageal squamous carcinoma cell line). *Materials and methods*: Cytotoxic activity was determined by the WST-1 assay. Different administration schedules of the two drugs were compared and evaluated for synergism, additivity, or antagonism with a quantitative method based on the median-effect principle of Chou and Talalay. Cell cycle perturbation and apoptosis were evaluated by flow cytometry. *Results*: Simultaneous treatment of cells with paclitaxel and oxaliplatin showed greater than additive effects. Upon 24-h sequential exposure, the sequence of paclitaxel followed by oxaliplatin showed synergistic effects in AZ-521 and HST-1 cells, and greater than additive effects in KSE-1 cells, while the opposite sequence yielded marked antagonistic effects in all three cell lines. Flow cytometric analysis indicated that paclitaxel induced G_2/M arrest with subsequent induction of apoptosis in the sub-G_1 phase. Apoptosis was most prominent when paclitaxel preceded oxaliplatin, which produced apoptosis in the majority of treated cells (75%). By contrast, the reverse sequence yielded only 39% induction of apoptotic cells, the rate being not different from those induced by each drug singly. *Conclusions*: Our findings suggest that the interaction of paclitaxel and oxaliplatin is highly schedule-dependent and that the sequential administration of paclitaxel followed by oxaliplatin should thus be incorporated into the design of a clinical trial.

Keywords Oxaliplatin · Paclitaxel · Drug interaction · Sequence-dependence

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Introduction

In view of the limited effectiveness of the currently available cytotoxic drugs for solid tumors such as gastric cancer, esophageal cancer, and head and neck cancer, there is an urgent need of new and better therapeutic approaches to improve the clinical outcome of these diseases. Multiple novel agents have been investigated in the treatment of patients with these cancers. Among these, paclitaxel and oxaliplatin are two anticancer drugs used increasingly in monotherapy or in combination with other drugs in the clinic.

Paclitaxel is a chemotherapeutic agent that induces apoptosis by arresting the cell cycle at the G_2/M phase through tubulin polymerization [28]. This agent has demonstrated clinical efficacy in the treatment of ovarian cancer, non-small-cell lung cancer, breast cancer, and head and neck cancer [26]. Paclitaxel produces peripheral neuropathy, dose-limiting bone marrow suppression, and alopecia [5]. Oxaliplatin (trans-l-1,2-diaminocyclohexane oxalato platinum II) is a third-generation platinum compound that acts as an alkylating agent, inhibiting DNA replication by forming adducts between two adjacent guanines or guanine and adenine [12]. Oxaliplatin has been demonstrated to exhibit antitumor activity against cell lines with acquired cisplatin resistance as well as clinical tumors that are intrinsically resistant to cisplatin and carboplatin [6, 9, 26]. Phase II studies of single-agent oxaliplatin have shown activity in colorectal [1], ovarian [23], breast [10], and untreated non-small-cell lung cancers [19]. Oxaliplatin has a different toxicity profile from that of cisplatin, with mild nausea and vomiting and, in contrast to

carboplatin, mild to moderate hematological toxicity. The dose-limiting toxicity of oxaliplatin is a dose-dependent and reversible peripheral neuropathy [7].

Paclitaxel has shown synergism with cisplatin both in vitro and in vivo [13, 18]. The combination of oxaliplatin and paclitaxel appears to show substantial activity in ovarian cancer patients previously treated with cisplatin or carboplatin, even in those with platinum resistance [8]. Although the combination of oxaliplatin and paclitaxel would be expected to have potent activity similar to that of the combination of paclitaxel and cisplatin, few preclinical data for the interaction between these drugs are currently available. In order to obtain the clinical rationale for the optimal administration schedule of this combination. we investigated the interaction between oxaliplatin and paclitaxel using an in vitro model of human cancer cell lines using a quantitative method which assessed the synergism or antagonism between these two agents.

Materials and methods

Cell lines and culture

The human AZ-521 gastric adenocarcinoma cell line was kindly provided by the JCRB Cell Bank (Tokyo, Japan) and the cells were maintained in Dulbecco's minimum essential medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand Ireland, N.Y.) in an incubator at 37°C and 100% humidity in air containing 5% CO_2. The human HST-1 tongue squamous carcinoma cell line [20] and the human KSE-1 esophageal squamous carcinoma cell line [17] were established in our laboratory and the cells were maintained under the same conditions as the AZ-521 cells.

Drugs

Paclitaxel was a gift from Bristol-Myers (Tokyo, Japan) and oxaliplatin was a gift from Yakult (Tokyo, Japan). Stock solutions of paclitaxel were prepared in DMSO and those of oxaliplatin were prepared in distilled water. Both solutions were stored at −4°C prior to use. The final concentration of DMSO for all experiments and treatments was maintained at less than 0.02%. These conditions were found to be non-cytotoxic.

Cytotoxicity assay

Cytotoxic activity was measured by the WST-1 assay (Wako Chemicals, Osaka, Japan) following the manufacturer's instructions [11]. The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of viable cells. Briefly, cells were plated into 96-well microtiter plates at a density of 5×10^3 cells/well, and incubated for 24 h for sufficient cell growth. Cells were then treated with graded concentrations of paclitaxel (0.3–1000 ng/ml) or oxaliplatin (0.3–1000 μg/ml) alone for 24 h, and were incubated with drug-free medium for an additional 24 h. Cells were washed with PBS and 100 μl medium, and 10 μl WST-1 solution was added to each well and the plates were incubated at 37°C for another 3 h. Absorbance at 450 nm and 640 nm was measured using a Delta Soft ELISA analysis program for Macintosh computers interfaced with a Bio-Tek microplate reader (Immuno-Mini NJ-2300). Wells containing only DMEM and WST-1 were used as controls. Each experiment was performed using six replicate wells for each drug concentration and carried out independently at least three times. The IC_{50} values were defined as the concentrations that reduced the absorbance in each test by 50%.

For the combination experiments, three different schemes were used to investigate the interaction of paclitaxel and oxaliplatin as shown in Fig. 1: in schedule A, paclitaxel and oxaliplatin were exposed simultaneously for 24 h and incubated for additional 24 h with drug-free medium; in schedule B, paclitaxel was administered for 24 h followed by oxaliplatin for 24 h; and in schedule C, oxaliplatin was administered for 24 h followed by paclitaxel. Immediately after these treatments, the cytotoxic effects were evaluated by WST-1 assay.

Analysis of combination effects

On the basis of the growth inhibition curve for each single drug, we analyzed the effects of the drug combinations using the method described by Chou and Talalay and the Calcusyn software program for automated analysis (Biosoft, Cambridge, United Kingdom) [2, 3]. The effect of combining the two drugs was evaluated by comparing the results of the sequential assays with those of the assays involving oxaliplatin or paclitaxel exposure

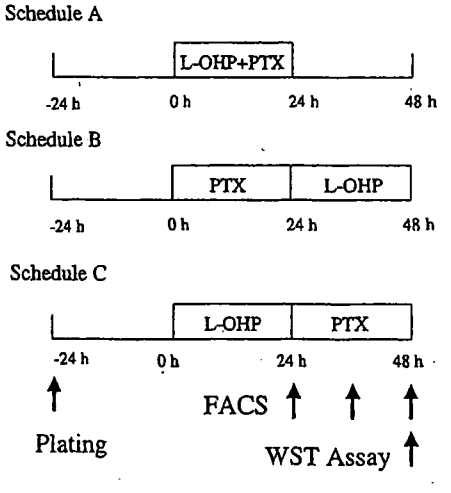

Fig. 1 The three combination schedules

alone. The combination effect was evaluated from iso-effect analysis (Cis), calculated as follows: $CI = C_{paclitaxel}/Cx_{paclitaxel} + C_{oxaliplatin}/Cx_{oxaliplatin}$, where $Cx_{paclitaxel}$ and $Cx_{oxaliplatin}$ are the concentrations of paclitaxel and oxaliplatin alone, respectively, needed to achieve a given effect (x%) and $C_{paclitaxel}$ and $C_{oxaliplatin}$ are the concentrations of paclitaxel and oxaliplatin needed for the same effect (x%) when the drugs are combined. These concentrations were calculated for each experiment and for each combination experiment at a fixed concentration ratio. The combination was considered as positive (synergistic) when the combination index was <1 and negative (antagonistic) when it was >1, and values of 1 were considered to indicate additivity.

Cell cycle determination

AZ-521 cells were cultured at 1×10^5 cells per 60-mm dish. The same protocols as described in the growth inhibition assay were used. After treatment, the cells were harvested, washed twice in ice-cold PBS (pH 7.4), and then fixed in 100% ethanol and stored at 4°C for up to 3 days prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were washed with PBS and stained with a solution containing propidium iodide and RNase (Sigma-Aldrich, St. Louis, Mo.) on ice for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACS/Calibur flow cytometer using the CELLQuest or ModFit 3.0 software packages (Becton Dickinson, San Jose, Calif.), and the percentages of apoptotic populations were determined by measuring the sub-G_1 phase using FACS analysis after collecting floating and trypsinized adherent cells at various times following drug exposure. Each experiment was performed in triplicate.

Results

Single-agent experiments

The cytotoxic activities of paclitaxel and oxaliplatin were tested individually on the three tumor cell lines. The cells were exposed to each drug for 24 h. The IC_{50} values (±SD) are summarized in Table 1. For paclitaxel, the IC_{50} ranged from 14.0 ng/ml (0.016 n *M*) for AZ-521 cells to 26.0 ng/ml (0.03 n *M*) for HST-1 cells. HST-1 cells were more resistant than AZ-521 or KSE-1 cells. AZ-521 cells were the most sensitive to oxaliplatin (0.95 µg/ml, 2.39 µ*M*) among the three tumor cell lines, and KSE-1 cells were the least sensitive (11.9 µg/ml, 29.7 µ*M*).

Median-effect analysis of paclitaxel and oxaliplatin combination in vitro

Paclitaxel and oxaliplatin were tested in different combinations to define the most effective schedule. Three different schedules were tested (simultaneous or sequential drug exposure as shown in Fig. 1) and the exposure time to each drug was 24 h. In AZ-521 cells, simultaneous treatment with the two drugs for 24 h caused largely additive effects in the moderate cytotoxic range (Fig. 2a). Sequential treatment with paclitaxel followed by oxaliplatin produced great synergy in all the ranges of cell kill fraction (CIFig. 2b). Conversely, when

Table 1 IC_{50} values of paclitaxel and oxaliplatin in three cell lines. Cells were treated with various concentrations of paclitaxel for 24 h or oxaliplatin for 24 h. The values are the means ± SD of three independent experiments

	AZ-521	HST-1	KSE-1
Paclitaxel (ng/ml)	14 ± 1.9	26 ± 2.6	18.9 ± 0.9
Oxaliplatin (µg/ml)	0.95 ± 0.4	3.5 ± 2.0	11.9 ± 5.6

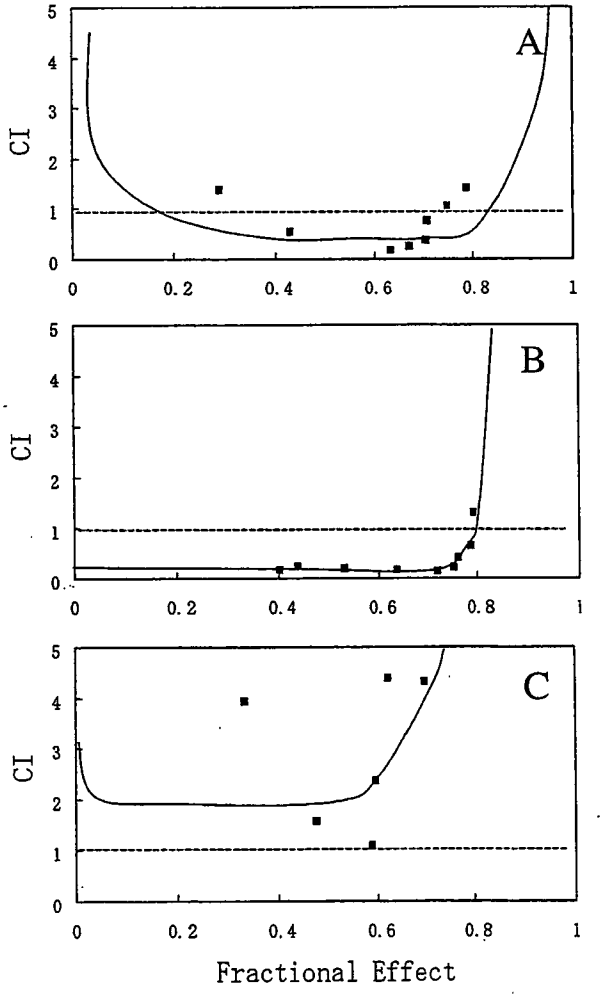

Fig. 2 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in AZ-521 cells. Cells were treated with (a) paclitaxel and oxaliplatin for 24 h simultaneously, (b) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (c) oxaliplatin for 24 h followed by paclitaxel for 24 h

the inverse sequence (oxaliplatin followed by paclitaxel) was used, antagonistic effects were observed at all levels of cell kill fraction (CI > 1; Fig. 2c).

In HST-1 cells, simultaneous treatment and sequence paclitaxel followed by oxaliplatin yielded similar synergistic effects at the same cytotoxic ranges corresponding to greater than 40% inhibition of cell growth (Fig.3a, b), whereas the opposite sequence showed antagonism (Fig. 3c). In the KSE-1 cells, simultaneous treatment and sequence paclitaxel followed by oxaliplatin showed additive to synergistic effects (Fig. 4a, b). In contrast, oxaliplatin followed by paclitaxel showed antagonistic effects (Fig. 4c).

Cell cycle perturbation and apoptosis

In an attempt to explain the mechanisms underlying the different types of interaction, the effects of paclitaxel and oxaliplatin on cell cycle distribution and apoptosis were studied in AZ-521 cells (Table 2). The cells were treated with these drugs either alone or in combination with different schedules, and cell cycle distribution was analyzed 24, 36 and 48 h after the beginning of treatment using flow cytometry. Paclitaxel alone at a dose of 12.5 ng/ml induced accumulation of cells in the G_2/M phase. At 1 μg/ml, oxaliplatin alone caused an increase in the G_1 population and a decrease in the S-phase population, showing that it inhibited G_1 to S progression. Treatment with paclitaxel prior to oxaliplatin induced accumulation of cell in the G_2/M phase as well as a reduction in the G_1 cell population, a similar distribution patterns to that observed in cells treated with paclitaxel alone, although a slight increase in the G_0/G_1 population and a decrease in the G_2/M population were observed compared with cells treated with paclitaxel alone. In contrast, oxaliplatin prior to paclitaxel caused almost identical distribution patterns to those observed

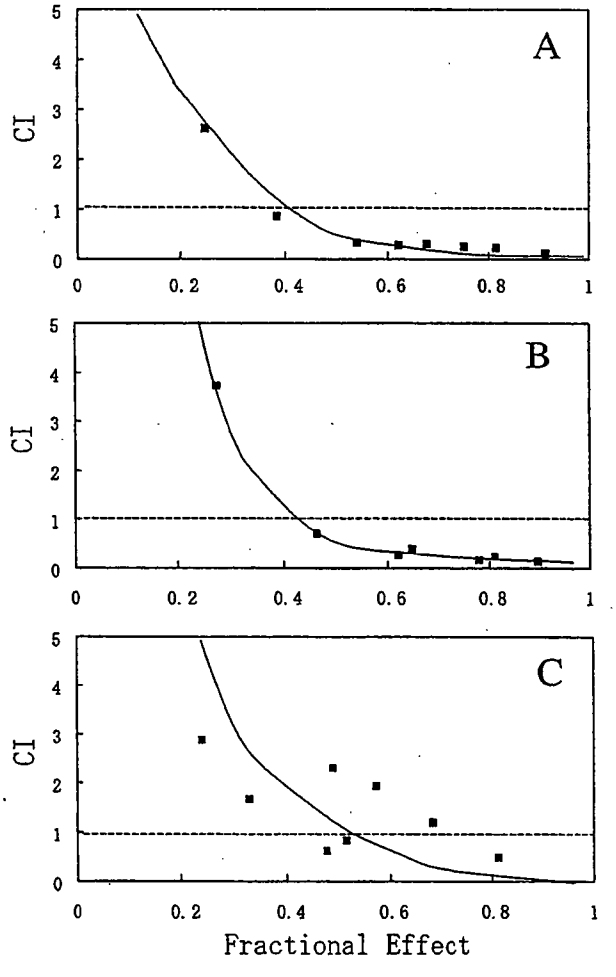

Fig. 3 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in HST-1 cells. Cells were treated with (a) paclitaxel and oxaliplatin for 24 h simultaneously, (b) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (c) oxaliplatin for 24 h followed by paclitaxel for 24 h

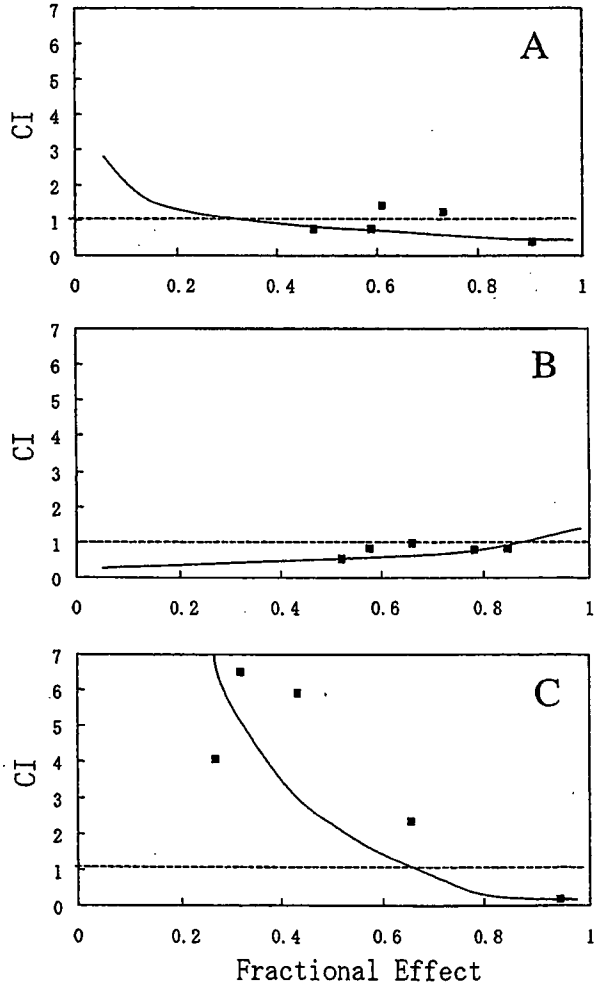

Fig. 4 Combination index (CI) plots of interactions between paclitaxel and oxaliplatin in KSE-1 cells. Cells were treated with (a) paclitaxel and oxaliplatin for 24 h simultaneously, (b) paclitaxel for 24 h followed by oxaliplatin for 24 h, or (c) oxaliplatin for 24 h followed by paclitaxel for 24 h