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Comparative study on various combination chemotherapies against human gastric cancer xenograft lines of well- and poorly-differentiated adenocarcinomas transplanted in nude mice

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Two human gastric cancer xenograft lines (GC-YN and GC-SF) transplanted in nude mice were employed to evaluate and compare the anticancer effect of seven single anticancer agents and their various combinations. Mitomycin C, cisplatin (Briplatin) (CDDP) and 5-fluorouracil (5-FU) were screened out to be effective against GC-YN and only epirubicin (Farmorubicin) (EPIR) was effective against GC-SF. Combinations of two of these 'effective' agents revealed that FP (5-FU + CDDP) is the most effective two-agent combination regimen against both lines, and some of those 'ineffective' single agents showed synergistic effects against both lines when combined with 5-FU. Moreover, three-agent combinations composed of FP and one of the other five agents were also evaluated to select out the most effective regimen. All the combinations showed higher inhibition on the tumor growth of GC-YN than FP regimen, and FP + adriamycin (Adriacin) (ADR) and FP + EPIR were more effective against GC-SF than FP. However, taking toxic effects into consideration, the results suggest that CDDP + 5-FU + EPIR (FPEPIR) may be the regimen most worthy of clinical trial in the chemotherapy against human gastric cancer.

Key words: Chemotherapy, gastric cancer, nude mouse.

Introduction

Although a major effort toward early diagnosis and surgery with curative intent has improved the survival rate of gastric cancer, the survival rates of patients with inoperable gastric cancer still remain low with a mean survival of less than 6 months.[1,2] Gastric cancer is now considered to be the cancer most relatively sensitive to chemotherapy as compared with other digestive organ cancers.[3,4] Extensive investigation has therefore been undertaken in recent years, either to develop new anticancer agents with improved therapeutic index[5,6] or to pursue more effective regimens.[4,7–13] For the chemotherapy of gastric cancer, 5-fluorouracil (5-FU), mitomycin C (MMC), and adriamycin (Adriacin) (ADR) have been conventionally applied and currently cisplatin (Briplatin) (CDDP), etoposide (Vepside) (VP-16) and epirubicin (Farmorubicin) (EPIR) have been newly applied. However, chemotherapy with a variety of single agents alone is of minimal practical benefit for gastric cancer patients, because they have shared the liability of low response rates and short duration of response.[14] Among various combination regimens, FAM (5-FU + ADR + MMC), which has been tested for more than 10 years, is one of the most extensively evaluated combination regimens. Although FAM produces a consistent response rate, like most single-agent regimens and two-agent combination regimens, it does not produce a complete response frequently.[14–17,18] With the development of new anticancer agents, however, oncologists have been encouraged to pursue more effective combination chemotherapeutic regimens.[18–22] The main purpose of this study is to pursue a safe and effective regimen that holds

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promise in clinical use. In this study, two histologically different human gastric cancer lines were employed to evaluate the efficacy of seven single agents, including three newly applied cancer agents— CDDP, VP-16, EPIR—and to establish a new beneficial combination regimen.

Materials and methods

Animals

Congenitally athymic male BALB/c nude (nu+/nu+) mice 6–10 weeks of age, were purchased from CLEA JAPAN, Tokyo, Japan. The mice were housed in Kyoto University Laboratory Animal Center under specific pathogen free (SPF) conditions.

Tumors

Two human gastric carcinoma lines (GC-YN and GC-SF), which were obtained from the resected specimens and established by transplanting in nude mice in our division, were employed. GC-YN is a well-differentiated tubular adenocarcinoma and GC-SF is a poorly differentiated adenocarcinoma. Their mean doubling time of tumor growth are 7.2 ± 0.23 days for GC-YN and 14.07 ± 1.89 days for GC-SF.

Anticancer agents

The anticancer agents and their dosages evaluated in this study are summarized in Table 1. The original solution of CDDP was used undiluted and VP-16 was diluted with 5% ethanol. All the other agents were dissolved or diluted with distilled water. All agents were administered via the intravenous route at a dosage of 4 times their respective maximal clinical dose (MCD) per kg of body weight; MCDs were approved by the Japanese Ministry of Health and Welfare. In mice, these doses approximate only half of the MCD per m^2 of body surface area.[23] The doses may be maximal for nude mice in three-agent combination regimens, because some combination regimens resulted in high mortalities in this study.

Drug testing protocol (Figure 1)

The tumors were cut into pieces of approx. 2–3 mm and were subcutaneously transplanted into the back of the mice. After transplantation, tumor length (L) and width (W) was serially measured with a caliper. The volume (V) of tumor and tumor growth rate (TGR) were calculated according to the following formulae:

$$V = L \times W^2/2, \quad TGR = V_n/V_0,$$

where V_n is the tumor volume estimated n days after initial administration, and V_0 is the tumor volume at the start of administration.

Treatment was initiated when tumors reached 100–300 mm^3 in volume, usually 2–3 weeks after transplantation. Tumor-bearing mice were randomized to groups of 5–6 animals each, including both treated and control groups. Single-agent, two-agent, and three-agent combinations were, respectively, evaluated in three successive experiments. Each experiment (Exp.) was repeated twice and the results were calculated from the accumulated data. In Exp. 1, each single agent was administered weekly on days 0, 7 and 14. In Exp. 2, two agents were administered alternatively on days 0, 3, 7 and 10. In Exp. 3, two agents were administered in the same way as in Exp. 2 and the third agent was given at half dose (i.e. 2 × MCD) on days 0, 3, 7 and 10. 5-FU was arbitrarily elected as the basic agent because it is the most completely evaluated

Table 1. Dose of anticancer agents evaluated against GC-YN and GC-SF (in mg)

Agent		MCD[a] (kg^{-1})	4 × MCD (kg^{-1})	MCD (m^{-2})
5-Fluorouracil	(5-FU^R: Kyowa Hakkou Ind., Tokyo, Japan)	10.0	40.0	80.0
Mitomycin	(MMC^R: Kyowa Hakkou Ind., Tokyo, Japan)	0.08	0.32	0.64
Adriamycin	(Adriacin^R: Kyowa Hakkou Ind., Tokyo, Japan)	0.8	3.2	6.4
Carboquone	(Esquinone^R: Sankyo Co. Ltd., Tokyo, Japan)	0.16	0.64	1.28
Cisplatin	(Briplatin^R: Bristol Myers Co. Ltd., Tokyo, Japan)	0.9	3.6	7.2
Etoposide	(Vepside^R: Bristol Myers Co. Ltd., Tokyo, Japan)	3.0	12.0	24.0
Epirubicin	(Farmorubicin^R: Farmitalia Carlo Erba Spa, Milan, Italy)	1.2	4.8	9.6

[a] Maximal clinical dose.

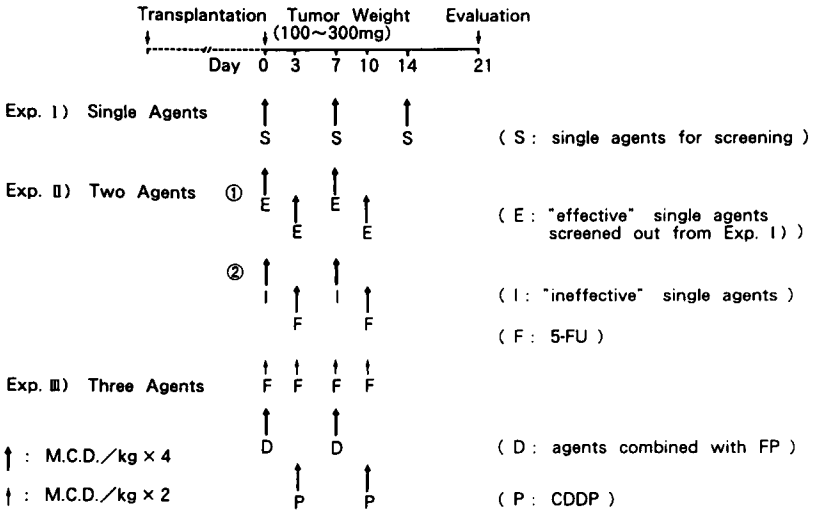

Figure 1. Protocols of single-agent chemotherapy and combination chemotherapies with two or three chemotherapeutic agents. In Exp. 1, seven single agents were screened for their effects against GC-YN and GC-SF. Each single agent was administered on day 0, 7 and 14, making a total dose of 12 times MCD/kg of body weight, and those which significantly inhibit tumor growth (i.e. $TGR_t < TGR_c$, $p < 0.05$) were defined as 'effective' single agents. In Exp. 2, the effects of two-agent combinations were evaluated and compared among (1) any combinations of two 'effective' single agents and/or (2) combinations of each of the 'ineffective' single agents and 5-FU, which is the most completely evaluated anticancer agent supposed to be effective for gastric cancer. In order to prevent severe toxicities, they were alternatively administered on days 0, 3, 7 and 10, making a total dose of 16 times MCD/kg, and the regimen showing the highest % inhibition was considered the most 'effective' two-agent combination. In Exp. 3, FP, which proved to be the most 'effective' two-agent combination, was combined with any of the remaining five agents (D). Any of the agents combining with FP as well as CDDP were administered on days 0, 3, 7 and 10, and 5-FU was given at a half dose on these 4 days, making a total dose of 24 times MCD/kg like FFF.

anticancer agent for gastric cancer.[14,24] In order to see and compare the effects of combination with 5-FU, 5-FU was repeatedly administered in Exp. 2 and Exp. 3 as FF (5-FU + 5-FU) and FFF (5-FU + 5-FU + 5-FU), of which the total doses administered approximate 1.3 and 2 times that given as a single agent in Exp. 1. The duration from the termination of treatment to the day of evaluation (day 21), i.e. the drug-free duration, of the combination chemotherapy in Exp. 2 and 3 is longer (11 days) than that of the single-agent chemotherapy in Exp. 1 (7 days). This may be important in regard to the 'rebound growth phenomenon'. The mice in the control group were administered with 0.1 ml of saline water. The efficacy of each anticancer regimen was evaluated 21 days after the start of treatment in terms of the % inhibition rate (*IR*) according to the following formula:

$$IR = (1 - TGR_t/TGR_c) \times 100\ (\%).$$

TGR_t and TGR_c indicate the TGR of the treated and control groups, respectively.

Toxicity was evaluated for 3 weeks from the start of treatment in terms of body weight ratio (W_n/W_0, where W_n is body weight measured n days after initial administration and W_0 is body weight at the start of administration) and mortality.

Statistics

All results were expressed as mean ± SD and p values were determined by Student's t-test using Medical Plan II computer software (Sankyo Co. Ltd.).

Results

The antitumor effects of single agents against GC-YN and GC-SF and the toxicities of single agents are summarized in Table 2. Among the single agents, CDDP, MMC and 5-FU are significantly effective against GC-YN with an *IR* of 67.6, 53.8 and 49.7%, respectively. By contrast, only EPIR is effective against GC-SF with an *IR* of 52.6%. No remarkable (<5%) loss of body weight or mortality was noted in groups of single agents.

The two-agent combinations were designed as follows: (1) combinations of independently effective single agents, and (2) 5-FU + other agents, because 5-FU is the agent most widely applied in combination chemotherapies for gastric cancer. Among the two-agent combinations (Table 3), FP (5-FU + CDDP) is the most effective regimen against GC-YN with an *IR* of 73.4% on day 21, followed by FQ (5-FU + carboquone (CQ)), 72.7%; FM (5-FU + MMC), 70.5%; FE (5-FU + VP-16), 67.5%; FA (5-FU + ADR), 62.6%; FEPIR (5-FU + EPIR), 60.3%; and MP (MMC + CDDP), 56.2%. In spite of a longer drug-free duration in the groups of combination chemotherapy, all of these two-agent combinations were effective against GC-YN ($p < 0.01$ vs control). FP is also the most effective two-agent regimen against GC-SF with an *IR* of 47.2%, followed by FQ, 45.2%; FEPIR, 44.8%; and FE, 44.0%. However, none of them surpassed the *IR* of EPIR alone. There was no remarkable toxicity in any group of two-agent combinations (Table 3). It is worthy to note that some 'ineffective' single agents (VP-16, ADR, CQ) showed synergistic effects on the growth of GC-YN when combined with 5-FU. Against GC-SF, combinations of two 'ineffective' agents (FP, FQ, FE) also showed synergistic effects.

Based on the effect of two-agent regimens, three-agent regimens were designed to include FP, and were compared with FAM which has been clinically used as a standard regimen for gastric cancer. Table 4 summarizes the antitumor effects and toxicities of three-agent combinations: FEP (5-FU + VP-16 + CDDP) has an *IR* of 90.7% on day 21, followed by FAP (5-FU + ADM + CDDP), 86.6%; FMP (5-FU + MMC + CDDP), 84.7%; FQP (5-FU + CQ + CDDP), 78.0%; and FPEPIR (5-FU + CDDP + EPIR), 76.4% against GC-YN. All of these three-agent combination regimens consisted of CDDP and 5-FU and had higher *IR*s than all the two-agent combination regimens. Against GC-SF, FPEPIR and FAP also showed significant inhibitions on tumor growth, although FAP did not

Table 2. Tumor inhibitory effect and toxicities of single agents on GC-YN and GC-SF

Protocol	Tumor inhibitory effect on				Toxicity	
	GC-YN		GC-SF		Body weight ratio (mean ± SD)	Mortality (%)
	TGR[a] (mean ± SD)	Mean *IR*[b] (%)	*TGR* (mean ± SD)	Mean *IR* (%)		
Control (saline)	7.87 ± 2.82 ($n = 16$)	—	2.48 ± 0.50 ($n = 12$)	—	1.02 ± 0.04 ($n = 28$)	0
MMC	3.13 ± 2.44* ($n = 6$)	53.8	2.81 ± 0.68 ($n = 6$)	−13.2	1.04 ± 0.05 ($n = 12$)	0
ADR	8.23 ± 2.98 ($n = 6$)	9.5	2.52 ± 0.60 ($n = 6$)	−1.5	1.01 ± 0.07 ($n = 12$)	0
EPIR	4.06 ± 1.22 ($n = 6$)	41.0	1.18 ± 0.40 ($n = 6$)	52.6	0.99 ± 0.03 ($n = 12$)	0
CQ	10.33 ± 4.67 ($n = 6$)	−14.9	3.75 ± 1.69 ($n = 6$)	−51.3	1.03 ± 0.07 ($n = 12$)	0
VP-16	6.37 ± 1.82 ($n = 6$)	17.7	3.54 ± 0.18 ($n = 6$)	−42.8	1.02 ± 0.03 ($n = 12$)	0
CDDP	1.94 ± 1.12*** ($n = 6$)	67.6	2.67 ± 0.76 ($n = 6$)	−7.8	0.98 ± 0.11 ($n = 12$)	0
5-FU	3.59 ± 1.39** ($n = 6$)	49.7	1.96 ± 0.32 ($n = 6$)	21.1	0.98 ± 0.03 ($n = 12$)	0

All these results were determined by the accumulated data from two experiments.
[a] *TGR*, tumor growth rate; [b] *IR*, inhibition rate.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (vs control).

Table 3. Tumor inhibitory effect and toxicities of two-agent combination on GC-YN and GC-SF

Protocol	Tumor inhibitory effect on				Toxicity	
	GC-YN		GC-SF		Body weight ratio (mean ± SD)	Mortality (%)
	TGR[a] (mean ± SD)	Mean *IR*[b] (%)	*TGR* (mean ± SD)	Mean *IR* (%)		
Control (saline)	8.67 ± 4.32 ($n = 15$)	—	2.48 ± 1.50 ($n = 10$)	—	1.03 ± 0.05 ($n = 25$)	0
MMC + CDDP[c]	3.80 ± 1.23 ($n = 6$)	56.2	Not evaluated	—	1.02 ± 0.05 ($n = 6$)	0
MMC + 5-FU[c]	2.56 ± 0.95*** ($n = 6$)	70.5	1.77 ± 0.96 ($n = 5$)	37.4	1.01 ± 0.03 ($n = 11$)	0
CDDP + 5-FU[c]	2.31 ± 0.68*** ($n = 6$)	73.4	1.31 ± 0.27* ($n = 5$)	47.2	0.98 ± 0.06 ($n = 11$)	
CQ + 5-FU[d]	2.37 ± 0.94*** ($n = 6$)	72.7	1.36 ± 0.14* ($n = 5$)	45.2	0.98 ± 0.04 ($n = 11$)	0
VP-16 + 5-FU[d]	2.82 ± 1.30*** ($n = 6$)	67.5	1.39 ± 0.26* ($n = 5$)	44.0	1.02 ± 0.06 ($n = 11$)	0
ADR + 5-FU[d]	3.24 ± 1.27*** ($n = 6$)	62.6	1.56 ± 0.44 ($n = 5$)	37.2	1.02 ± 0.02 ($n = 11$)	
EPIR + 5-FU[d]	3.44 ± 1.16** ($n = 6$)	60.3	1.37 ± 0.28* ($n = 5$)	44.8	1.00 ± 0.10 ($n = 11$)	0
5-FU + 5-FU[e]	1.53 ± 0.29*** ($n = 7$, 3/7 died)	77.1	Not evaluated	—	0.92 ± 0.06 ($n = 4$)	43 (3/7)

All these results were determined by the accumulated data from two experiments.
[a] *TGR*, tumor growth rate; [b] *IR*, inhibition rate.
[c] Combinations of two 'effective' agents against GC-YN.
[d] Combinations of 5-FU and 'ineffective' agents for GC-YN.
[e] 5-FU administered twice a week for 2 weeks, of which the total dose approximates 1.3 times of that administered as a single agent, was evaluated as a contrast for two-agent combinations.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (vs control).

surpass EPIR alone in the antitumor effect. Except for the regimens of FPEPIR and FQP, these three-agent combination regimens also had quite severe toxicities (Table 4): FEP showed a significant (about 18%) loss of body weight, and a mortality rate of 21.4%; FAP had a mortality rate of 21.4%; and FMP had a mortality rate of 7.1%. FQP and FPEPIR showed no significant loss of body weight, and no mortality. In contrast, FAM had either a low *IR* and/or severe side effects, and because of high mortality (>70%) on day 21, its anticancer effect against GC-YN was evaluated on day 17, with an *IR* of 66.1%, about 15% loss of body weight and a mortality rate over 50%. It is worthy to note that FF and FFF also had remarkable inhibitions on tumor growth; however, their toxicities were too severe to be accepted.

Discussion

In this study, GC-YN is sensitive to MMC, 5-FU and CDDP, and GC-SF is sensitive to EPIR alone. In two-agent combination regimens, however, FP, FQ, FE and FEPIR were effective against both GC-YN and GC-SF. In three-agent regimens, FAP and FPEPIR were effective against both lines. Table 5 reviews the previously published results of single-agent chemotherapy in advanced gastric cancer.[2,3,24–29] 5-FU, MMC and CDDP are reported to be comparatively effective against gastric cancers.[2,3,24,26] In this study, GC-YN is sensitive to these agents and GC-SF is resistant to them. Numerous attempts have been made to develop effective combination chemotherapeutic regimens. The currently published results of combination chemotherapeutic regimens tested in patients with gastric cancer are summarized in Table 6.[7,9,12,14–16,26,30–35] FP, FAP and FAM are clinically effective regimens,[9,14,15,33,35] and in terms of tumor inhibitory effects alone, the results of this study seem to be consistent with these reports. However, if side effects are also taken into consideration, only FPEPIR is shown to be effective and a safe regimen worthy of clinical application. FAM, which had been used as one of the standard combination

Table 4. Tumor inhibitory effect and toxicities of three-agent combination on GC-YN and GC-SF

Protocol	Tumor inhibitory effect on				Toxicity	
	GC-YN		GC-SF		Body weight ratio (mean ± SD)	Mortality (%)
	TGR[a] (mean ± SD)	Mean *IR*[b] (%)	*TGR* (mean ± SD)	Mean *IR* (%)		
Control (saline)	8.12 ± 4.56 ($n = 15$)	—	2.46 ± 0.06 ($n = 10$)	—	1.00 ± 0.08 ($n = 25$)	0
FP + MMC	1.17 ± 0.35** ($n = 8$, 1/8 died)	84.7	1.82 ± 0.72 ($n = 6$)	31.6	0.97 ± 0.11 ($n = 13$)	7.1 (1/14)
FP + CQ	1.62 ± 0.72** ($n = 7$)	80.0	1.58 ± 0.35 ($n = 6$)	36.4	0.99 ± 0.07 ($n = 13$)	0
FP + VP-16	0.71 ± 0.30** ($n = 8$, 3/8 died)	90.7	1.62 ± 0.34 ($n = 6$)	34.8	0.82 ± 0.06* ($n = 11$)	21.4 (3/14)
FP + ADR	1.03 ± 0.28** ($n = 8$, 3/8 died)	86.6	1.22 ± 0.19 ($n = 6$)	50.9	1.13 ± 0.15* ($n = 11$)	21.4 (3/14)
FP + EPIR	1.82 ± 0.74* ($n = 8$)	76.1	1.10 ± 0.15 ($n = 6$)	55.6	1.01 ± 0.05 ($n = 14$)	0
FAM[c]	2.49 ± 0.05 ($n = 5$, 3/5 died)	66.1	Not evaluated	—	0.85 ± 0.07 ($n = 2$)	60 (3/5)
FFF[d]	1.15 ± 0.24 ($n = 7$, all died)	72.6	Not evaluated	—	0.92 ± 0.03 ($n = 7$)	100 (7/7)

All these results were determined by the accumulated data from two experiments.
[a] *TGR*, tumor growth rate; [b] *IR*, inhibition rate.
[c] FAM was evaluated on day 17 because of severe toxicity and high mortality.
[d] FFF was evaluated on day 14 because no mice survived beyond day 14.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (vs control).

regimens for years,[14–16] had low efficacy against GC-YN and had severe toxicity also. FAP, which has been currently applied in clinical chemotherapy of gastric cancer[7,32–34], also showed high mortality rates in this study. FQP, FMP and FEP are effective against GC-YN but not effective against GC-SF. These shortcomings may be responsible for the low complete response rate in the clinical reports.[14] Taken together, we suggest that three-agent combination regimens are more worthy of clinical trial than two-agent combination regimens or single agents, and among the three-agent combination regimens evaluated in this study, FPEPIR may be the regimen of choice in patients with advanced gastric cancer. The relatively high response rate of EPIR as a single agent,[28,29] and its relatively low toxicity may be responsible for the efficacy of the FPEPIR regimen. We are encouraged, if not fully convinced, by the temporary result of the early phase II study on the FPEPIR regimen (CDDP, 50 mg/body day 1; EPIR, 40 mg/m^2 day 2; 5-FU, 250 mg/body days 2–5, q 2 weeks) for various advanced solid tumors, especially for gastric cancer, which is being undertaken in our department, with a response rate of 38% (3/8, one complete response and two partial responses) for gastric cancers as revealed in the interim report (in preparation). According to this result, Kyoto Research Group for Chemotherapy of Gastric Cancer has started a randomized clinical study on the comparison of FP and FPEPIR. The results of this study will be reported in the near future. In-vivo studies using human gastric lines of different histology or

Table 5. Single-agent chemotherapy in advanced gastric cancer

Drug	No. of responses/ No. of patients	Response (%)	Reference
5-FU	84/392	21	24
MMC	63/211	30	24
	90/398	23	2*
ADR	17/68	25	25, 26
	9/62	15	2*
CDDP	10/49	20	3,27
	32/144	21	2*
EPIR	7/47	15	28, 29

* Japanese Reports.

Table 6. Combination chemotherapy for advanced gastric cancer

	Combination	No. of responses/ No. of patients	Response (%)	Median duration of survival/response (months)	Reference
Two agents	5-FU + CDDP(FP)	0/13	0	2.5 /	12
		21/53	40 (2% CR, 38% PR)	/ 9 (2–31)	9
	Overall	21/66	32		
	5-FU + MMC (FM)	6/43	14	6.0 /	30
		17/53	32	4.0 /	26
	Overall	23/96	24		
	5-FU + ADR (FA)	3/11	27	7.0 /	31
		1/19	5	6.0 /	32
	Overall	4/30	13		
Three agents	5-FU + ADR + MMC	18/36	50	5.5 / 9.5	14
	(FAM)	28/81	35 (5% CR, 30% PR)	17.0[a]/10.5	15
		18/46	39 (4% CR, 35% PR)	6.4 /	16
	Overall	64/163	39 (4% CR, 35% PR)		
	5-FU + ADR + CDDP	10/35	29	5.5 /	7
	(FAP)	9/18	50 (6% CR, 44% PR)	/ 4.5	33
		9/18	50	12.0 /6.0	34
		13/26	50 (11% CR, 39% PR)	9.0 /	35
	Overall	41/97	42 (7% CR, 35% PR)		

CR, complete response; PR, partial response.
[a] Survival duration in responders only.

different degrees of differentiation such as moderately or poorly differentiated adenocarcinomas, may be necessary to confirm the effectiveness of various chemotherapies. In this study, GC-YN and GC-SF showed different chemosensitivities to single agents, suggesting that these lines may be feasible for screening the combination regimens, which may be commonly effective against human gastric cancers with different degrees of differentiation. Our study also demonstrated that xenotransplantation of human cancers in nude mice can provide a useful in-vivo model for tumor-oriented trials which are feasible with the hope of clinical applicability.[36–39]

Although using the nude mouse system for pursuing effective regimens, particularly for the combination regimens, is quite expensive and time-wasting, we believe that this may be the only way to predict which regimen will hold the best promise for clinical trials. It may be difficult to evaluate or compare every combination of even the most commonly used anticancer agents. However, this study suggests that the 'optimal' combinations may enhance the efficacy of each individual agent, reduce unnecessary toxicity, and improve the response rates or the complete response rates. Finally, we hope that the FPEPIR regimen may reduce the incidence of recurrence or refractoriness and improve survival rate.

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