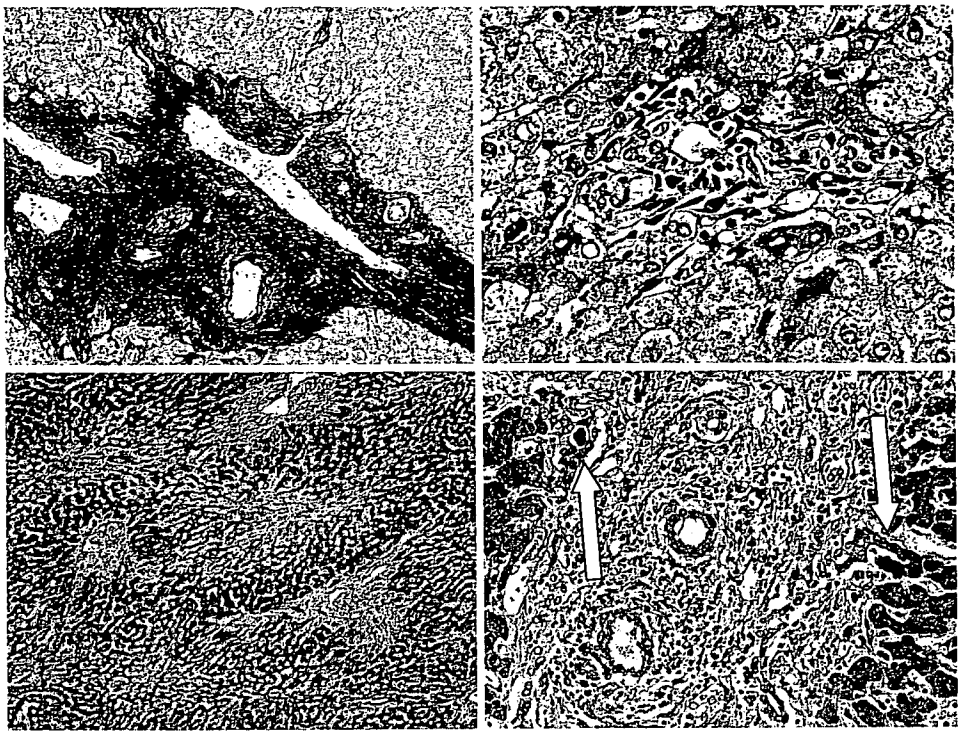

図 2

手術標本の非腫瘍部
a: 葉間結合織，門脈の内膜が肥厚しており門脈圧亢進を認める。
b: 胆管上皮が腫大し内腔を閉塞している。
肝針生検標本
c: 小葉間組織に架橋線維を認める。
d: Herring 管が障害されて胆汁栓が詰まっている（矢印）。

表 1 主要肝機能検査

	入院時	手術前	手術後	再入院時	肝生検時	術後91日
T-Bil (mg/dL)	0.4	0.3	0.8	3.1	3.4	14.8
GOT (IU/L)	15	17	28	30	34	113
GPT (IU/L)	6	5	31	31	28	122
Ch-E (IU/L)	298	274	100	87	78	66
Alb (g/dL)	3	3.9	2.2	3.6	1.5	1.9
Plt ($\times 10^4/mm^3$)	23.2	12.4	15.3	17.1	20.6	1.4

考え，肝切除を優先して二期的に肺切除を予定した。

手術: 2005年12月7日，肝右葉切除術を施行した。術中所見では肝右葉の主転移巣の大部分は壊死性変化を呈しており，その他の小転移巣は瘢痕化していた。切除標本の病理組織所見では肝転移巣の大部分は壊死しており，壊死組織のなかに高～中分化腺癌が小結節状に散在していた。また，主転移巣以外にも小転移巣をいくつか認め，それらの一部は線維化して瘢痕組織に置き換わっていた。非腫瘍部では微小胆管の閉塞，門脈圧亢進を示す大小不同の門脈径と門脈内膜の肥厚を認めた（図2）。経過良好にて術後19日に退院となり，二期的に肺転移の切除を計画していた。しかし術後28日，肝機能低下を認め再入院となった。その後，肝障害は進行し（表1），門脈造影検査にて門脈の拡張と血流のうっ滞，腹部CTにて脾腫，腹水貯留を認め肝不全へ移行した。術後61日に施行した肝針生検の病理所見（図2）では微小胆管の閉塞，小葉間組織の線維化，大小不同の門脈径と門脈内膜肥厚，さらにHerring管が障害されており胆汁栓を認めた。FFP投与など残肝救済を試みたが，術後95日に肝不全により死亡した。

III. 考　察

海外では切除不能，もしくは肝外病変を伴う大腸癌遠隔転移に対して積極的にneoadjuvant FOLFOXを行い，切除率を向上させている[5,6]，一方oxaliplatinによる肝臓の毛細血管の変性や脂肪蓄積について報告されている[3]。Adamら[7]によればneoadjuvant FOLFOXを行い切除した肝組織の病理所見は，LV/5-FU症例のそれよ

りも線維化，脂肪蓄積所見を多く認めたと報告している。また oxaliplatin＋capecitabine を使用し肝毛細血管の閉塞と線維化を認め，門脈圧亢進症を合併して肝不全で死亡した1例が報告されている[8]。さらに Rubbia-Brandt ら[9]によれば，大腸癌肝転移153例のうち化学療法後に肝切除した87例中21例（24%）の肝非腫瘍部の病理組織標本において毛細血管の静脈閉塞と小葉間組織の線維化を認め，一方，化学療法を施行せず肝切除を行った66例の肝非腫瘍部には同様の所見は認めなかった。このうち化学療法を施行した症例の78%が oxaliplatin を含むレジメンで，oxaliplatin による毛細血管壁の破裂とコラーゲンの堆積，そして線維化が肝障害の原因であると報告している。

本症例は Child-Turcotte 分類 A，ICG R_{15}分値 10%以下，CT volumetry で残肝容積を十分確認して手術に望んだ肝硬変のない正常肝症例であり，術中のトラブルはなく手術操作による肝不全は考えにくい。再入院時に肝針生検で得た残肝組織標本で微小胆管の閉塞，門脈圧亢進，小葉間組織の線維化を認めたが手術標本の非腫瘍部でも同様な所見を認めており，つまり手術時には肝障害は存在していたことになる。また，薬剤性の障害因子に弱い胆管と肝細胞索の移行部である Herring 管が障害されており，mFOLFOX 6 により障害を受けた肝臓が肝切除を機に肝不全へ移行した可能性が示唆される。今後，切除不能肝転移に対して切除を視野に入れた neoadjuvant FOLFOX の増加が予想される。oxaliplatin に肝臓が暴露している時間が長いほど肝障害が強まる[10]ことより，長期間の neoadjuvant FOLFOX を施行した場合，肝障害に対する画像診断の確立が急務であり，必要であれば肝生検[3]を行い非腫瘍部の肝組織の状態を観察することが重要である。もし，これらにより重度の肝障害を認めた場合，たとえ肝転移巣が切除可能の状態にあっても手術適応を慎重に考えるべきである。

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長期生存を得ている胃癌術後肝転移症例の1例

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A Long-Term Survival Case of Metachronous Liver Metastasis from Gastric Cancer: Sou Katayanagi[*1], Yu Takagi[*1], Hideo Sudo[*1], Takeshi Suda[*1], Sumito Hoshino[*1], Kazushige Itoh[*1], Akihiko Tsuchida[*1], Tatsuya Aoki[*1], Takashi Kawai[*2] and Tetsuya Yamagishi[*2] ([*1]*Dept. of Surgery and* [*2]*Endoscopic Center, Tokyo Medical University*)

Summary

A 62-year-old man was admitted for gastric cancer. He was performed a distal gastrectomy with Billroth I reconstruction in August 1999. Then he had remnant gastric cancer and metachronous liver cancer in November 2002. He was performed a total gastrectomy and partial hepatic resection. The histological findings of remnant stomach and liver cancer showed a same pattern of the primary gastric cancer.

Another metachronous liver cancer appeared in March 2006. He was treated with chemotherapy using S-1 (day 1-21) and CDDP 20 mg/m^2 (day 1, 8 and 15) q5w. The size of liver metastasis was kept the same for 16 months. Key words: Gastric cancer, Liver metastasis, Metachronous

要旨 症例は62歳，男性。1999年8月に胃癌にて幽門側胃切除術，D2郭清，Billroth I法再建術を施行した。2002年11月に残胃癌（tub 2）および肝転移を認めたため残胃全摘術，肝部分切除術を施行した。病理所見より残胃および肝転移は初回胃癌の異時性転移と考えられた。2006年3月肝S7に3cm大の新規の肝転移巣を認めたため，4月よりS-1（day 1～21）＋CDDP 20 mg/m^2 (day 1, 8, 15) q5wを開始した。現在11コースまで施行しているが，新規肝転移の出現はなくSD継続中である。

はじめに

胃癌肝転移症例は予後不良であるが，今回われわれは初回肝転移より肝切除および化学療法により，4年8か月の長期生存を得られている症例を経験したので報告する。

I. 症 例

患者: 62歳，男性。

既往歴: 特記すべきことなし。

家族歴: 特記すべきことなし。

経過: 1999年8月にM領域前壁の胃癌にて幽門側胃切除術，D2郭清，Billroth I法再建術を施行した。病理組織学的所見ではM-Ant, 0-III+IIc, 2×1.5 cm, tub 1-tub 2, PM(−), DM(−), p-T2(MP)N1H0P0CY0M0, Stage II, ly3, v3, 根治度Aであった。

病理組織学的所見は核小体が明瞭で，クロマチンの粗大もみられ，類円形の核をもちN/C比も高かった。腺管内は壊死物を伴いcribriform pattern（篩状構造）を呈しておりtub 2と診断した。また，先進部では一部腺管の乱れもありpor 2の構造も認められた。術後補助化学療法として2001年1月まで5-FUを内服した。

2001年9月CA19-9が上昇したためFP low-dose(5-FU 500 mg/body day 1～14, CDDP 10 mg/body day 1～5, 8～12）による化学療法施行後，UFTの内服を開始した。2002年11月（術後3年3か月）にCA19-9が再上昇したため精査し，残胃癌（tub2）および肝転移を認めたため残胃全摘術，肝外側区域切除術を施行した。M-3-S, 0-IIc, tub 2, PM（−），DM（−），p-T1 (SM2) N0H0P0CYxM0, Stage IA, ly1, v0であった。病理組織学的所見は残胃粘膜に病変は，はっきりせず腸上皮化成を主体としており粘膜下層のlevelで太い脈管

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片柳 創

0385-0684/07/¥500/論文/JCLS

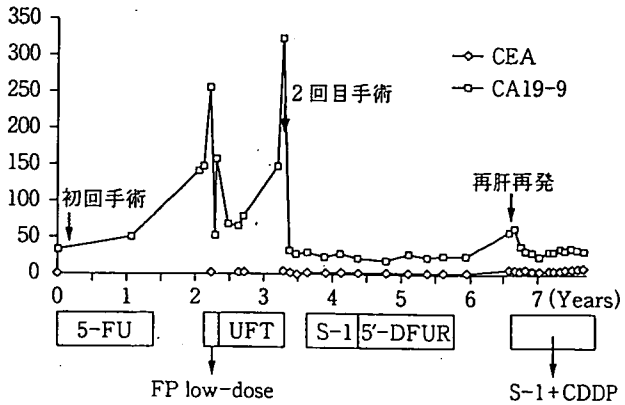
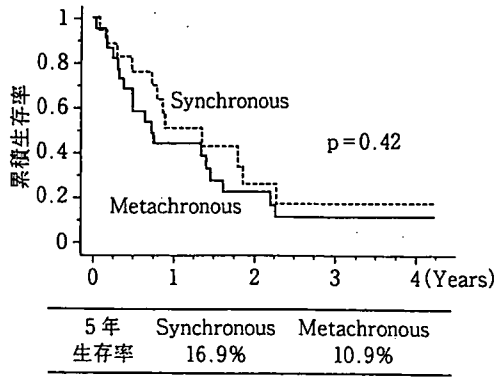


図1 治療経過



5年生存率	Synchronous	Metachronous
	16.9%	10.9%

図2 当科における肝転移症例の予後
手術後に異時性肝転移出現するまでのDFS: 507日 (median)

に cribriform pattern 様の侵襲を認めた。肝臓は境界明瞭で結節状に増殖し同様に cribriform pattern を呈した腫瘍を認めた。以上より，今回の腫瘍は初回の胃癌の異時性残胃および肝転移と判断した。

2003年3月より術後補助化学療法として S-1 の内服を開始，12月より2004年12月まで5'-DFUR を内服した。その後は休薬していたが，2006年3月に肝S7に3 cm 大の新規の肝転移巣を認めた。患者の希望により，手術ではなく2006年4月より S-1 (day 1～21) +CDDP 20 mg/m^2 (day 1, 8, 15) q5w を開始，現在11コースまで施行しているが新規肝転移の出現はなく，SD 継続中で，初回肝転移より4年8か月の長期生存中である(図1)。

II. 考　察

胃癌治療ガイドラインでは，胃癌肝転移（同時性，異時性）に対する肝切除の有効性についての評価は確立されていない[1]。胃癌肝転移症例は診断時に両葉に転移していることも多く，また腹膜転移やリンパ節転移などの非治癒因子を伴っていることも多く非切除になり，化学療法となる症例が多い。このため，根治度Bの切除は全体の10%しか施行されていない[2]。

外科的肝切除の適応は単発がよいとされている[3-5]が，複数個の転移巣が存在していても肝切除によって長期生存が得られる場合もあるため[3,4,6,7]，根治度Bが得られる場合が適応との報告もある[8]。転移個数に関しては画像診断によって大きく異なってくる可能性が高く，より精度の高い診断が必要であると思われる。

同時性肝転移に対する肝切除は5年生存率0～33.8%[4,7,9-11]，異時性肝転移に対しても25～30%[7,12]と，長期生存が得られることも少なくない。転移時期による差は認めないのが現状であると考える。

当科では，基本的に S-1 が認可された1999年以降は胃癌肝転移症例に対しては，単発で他の非治癒因子がない症例以外は手術を施行していない。基本的に食事摂取可能であれば S-1 based の化学療法を施行しており，その5年生存率は同時性で16.9%，異時性で10.9%と比較的良好である（図2)。2007年の ASCO にて切除不能進行・再発胃癌に対して S-1+CDDP 群が MST 13か月，S-1単独群が11か月と有意に予後を改善する[13]との報告もあり，今後さらに化学療法により予後が延長されていく可能性が高いと考えられた。

本症例は初回手術後に14か月間5-FU を内服し休薬したところ，8か月後に CA19-9 の上昇を認めたため化学療法を施行し軽快した。2回目の手術後に20か月間5-FU を内服し休薬したところ，16か月後に肝再発を来しており，5-FU 系の薬剤が補助化学療法として有効であった可能性が考えられた。

われわれは，胃癌術後の異時性肝転移に対して PEIT および動注リザーバー併用化学療法を施行し50%生存期間が877日と良好な成績を得ており，治療の選択肢の一つになる可能性があると考えられた[14]。

胃癌肝転移に対する治療はまだ確立されていない。胃癌に対する化学療法の成績が大腸癌と同様に向上してきており，今後もさらに改善されることが期待される。しかし，手術により長期生存する症例も少数例ではあるが存在している。今後，多施設共同研究により RCT を施行し，なるべく早く EBM を構築するのが急務である。

本論文の要旨は第29回日本癌局所療法研究会において発表した。

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Pretreatment with S-1, an Oral Derivative of 5-Fluorouracil, Enhances Gemcitabine Effects in Pancreatic Cancer Xenografts

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Abstract. *Background: The systemic administration of gemcitabine (GEM) has been accepted as a standard treatment for patients with advanced pancreatic cancer. The major mediator of cellular uptake of GEM is the human equilibrative nucleoside transporter 1 (hENT1) whose expression is up-regulated by thymidylate synthase inhibitors, such as 5-fluorouracil (5-FU). S-1 is a novel oral derivative of the 5-FU prodrug tegafur combined with two modulators. Recent clinical trials have reported the promising effect of S-1 in pancreatic cancer. The purpose of this study was to evaluate the relationship between different schedules and the effects of GEM/S-1 combination therapy on pancreatic cancer xenograft models. Materials and Methods: Human pancreatic tumor xenografts were prepared by subcutaneous implantation of MiaPaCa-2 into nude mice. Expression of hENT1 was determined by quantitative RT-PCR. GEM cellular uptake was determined using [^{3}H] GEM. Results: Significant increases in hENT1 expression and GEM cellular uptake were observed after S-1 treatment. Six different treatment schedules (no treatment, single agent of GEM or S-1, combination treatment with GEM either before, simultaneously or following administration of S-1) were compared. Significant tumor growth inhibition was observed in the mice treated with S-1 followed by GEM compared to either untreated mice or the mice treated with the other schedules. Conclusion: Based on the effects of S-1 on the uptake of GEM, S-1 should be used before GEM treatment. The GEM/S-1 combination therapy in patients with pancreatic cancer may be promising and should be tested in clinical trials.*

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Gemcitabine (2',2'-difluorodeoxycytidine (dFdC); Gemzar), accepted as a standard chemotherapeutic agent for patients with advanced pancreatic cancer, is a cell cycle-dependent deoxycytidine analogue of the antimetabolic class. It must first be transported into the cell and then be phosphorylated to its active triphosphate form. Incorporation of gemcitabine triphosphate into DNA is most likely the major mechanism through which gemcitabine exerts its cytotoxic actions (1).

Cells can synthesize nucleotides either through a *de novo* synthesis pathway or a salvage pathway. 5-Fluorouracil (5-FU), one of the thymidylate synthase (TS) inhibitors, is known to act as a *de novo* synthesis inhibitor (2). In the salvage pathway, nucleosides and nucleobases must first be transported across the cell membrane by nucleoside transporter proteins. In addition to nucleosides, nucleoside analogues are also taken up into the cell *via* these specific transporters (3). Gemcitabine is a substrate for five of the nucleoside transporters found in humans (4). These are human equilibrative nucleoside transporter 1 (hENT1), hENT2, human concentrative nucleoside transporter 1 (hCNT1), hCNT2 and hCNT3. The most active gemcitabine uptake is *via* hENT1 (4). It has been reported that 5-FU leads to an increase in cell surface hENT1 (5, 6). An increase in hENT1 can potentially augment the effect of gemcitabine because this agent enters the cell *via* hENT1. In fact, it has also been reported that pretreatment of pancreatic cancer cell lines *in vitro* and *in vivo* with 5-FU augmented the effects of single-agent gemcitabine

treatment, whereas concurrent treatment or gemcitabine prior to 5-FU did not (7, 8). These results suggest that the effect of gemcitabine/5-FU combination therapy could be dependent on the selected treatment schedule.

S-1 (TS-1) is an oral fluorinated pyrimidine which contains tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (Oxo) in a molar ratio of FT:CDHP:Oxo of 1:0.4:1, based on the biochemical modulation of 5-FU (9, 10). FT, a prodrug of 5-FU, is gradually converted to 5-FU and is rapidly catabolized by dihydropyrimidine dehydrogenase (DPD) in the liver. CDHP is a competitive inhibitor of 5-FU catabolism, being about 180 times more potent than uracil in inhibiting DPD (11). When combined with 5-FU, this results in the prolonged maintenance of 5-FU concentrations, both in plasma and in tumors. In addition, it has been suggested that CDHP has the potential to enhance the antitumor activity of 5-FU against subcutaneous tumors in nude mice, using human pancreatic carcinoma cells with a high tumoral DPD activity (12). Oxo is a selective inhibitor of phosphoribosyl pyrophosphate transferase in normal gastrointestinal tissues, resulting in decreased drug incorporation into cellular RNA and, therefore, in the reduction of gastrointestinal toxicity (13). Recent Phase II clinical trials using S-1 as a single agent have shown promising results in various solid tumors, particularly gastric and colorectal cancers, with a response rate of 31.6 to 53.6% and 16.7 to 39.5%, respectively (14). In patients with metastatic pancreatic cancer, a Phase II clinical trial showed the safety and efficacy of S-1, with a response rate of 37.5% and a median survival time of 8.8 months (15).

The relatively mild toxicity profile of gemcitabine has allowed for the development of gemcitabine-based combination chemotherapy regimens (16, 17). The combination of gemcitabine and 5-FU has been shown to have a marked synergistic cytotoxic effect including against pancreatic carcinoma cells in an *in vitro* assay (18, 19). The administration of oral S-1 is more convenient and simulates the effect of continuous infusion of 5-FU. However, no randomized Phase III trial has yet established the survival benefits of a combination of gemcitabine and 5-FU compared to gemcitabine alone (16, 17). Recently, two clinical trials of gemcitabine and S-1 have demonstrated favorable response and tolerability with different dose and schedule (20, 21). Therefore, we focused on optimizing the efficacy of gemcitabine through modification of the schedule of these two agents.

The purpose of the present study was to evaluate the relationship between the schedules of gemcitabine/S-1 combination therapy and their effects in pancreatic cancer. We hypothesized that pretreatment with S-1 would increase hENT1 and thus increase the cytotoxicity of gemcitabine, which enters the cell *via* hENT1, in pancreatic cancer.

Materials and Methods

Cell culture. In a previous study, we selected three human pancreatic carcinoma cell lines which showed a higher rate of 5-FU-induced increase in hENT1 mRNA, however, we failed to establish subcutaneous tumors except for MiaPaCa-2, hence the human pancreatic carcinoma cell line MiaPaCa-2 was used in the present study (8). MiaPaCa-2 was obtained from the Japanese Collection of Research Bioresources (JCRB, Japan) and was cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Rockville, MD, USA) and 100 units/ml each of penicillin and streptomycin.

Drugs and reagents. S-1 (TS-1) and gemcitabine (GEMZAR) were kindly provided by Taiho Pharmaceutical Co., Ltd (Tokyo, Japan) and Eli Lily, Japan (Indianapolis, IN, USA) respectively. [^{3}H] Gemcitabine was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). All drugs were dissolved in distilled water.

Pancreatic cancer xenograft model and treatment. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Medicine, Osaka University. Four-week-old female BALB/c *nu/nu* mice were purchased from Japan Clea (Tokyo, Japan) and maintained in specific pathogen-free conditions. Human pancreatic tumor xenografts were prepared by subcutaneous implantation of MiaPaCa-2 cells ($5x10^6$ cells/ 100 μl phosphate-buffered saline) into the right back of nude mice. When the tumors reached a volume between 100 and 200 mm^3, mice were randomly divided into treatment groups (n=5 for each group) according to tumor volume (day 0). S-1 was orally administrated at a dose of 10 mg/kg/day as described elsewhere (22), and gemcitabine was injected into the peritoneal cavity at a dose of 240 mg/kg as described elsewhere (23).

Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR). RNA extraction was carried out with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) using a single-step method, and cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), as described elsewhere (24). In this assay, porphobilinogen deaminase (PBGD) mRNA was used as an internal control (25). Q-RT-PCR was performed in a LightCycler apparatus using LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The PCR primers used for detection of PBGD and hENT1 cDNAs were synthesized as described elsewhere (25, 26). PCR was performed with cycling conditions of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 62°C for 10 s, and extension at 72°C for 20 s. To quantify the data, hENT1 mRNA levels were normalized by PBGD mRNA levels.

hENT1 mRNA expression and [^{3}H] gemcitabine uptake assay in vivo. The BALB/c mice bearing distinct MiaPaCa-2 tumors were divided into the following three groups of 6 mice each: (a) no treatment; (b) daily oral administration of S-1 for 5 consecutive days at a dose of 10 mg/kg/day (day 1 to 5); (c) daily oral administration of S-1 for 5 consecutive days at the dose of 50 mg/kg/day (days 1 to 5). On day 6, MiaPaCa-2 tumors in three of the mice killed by cervical dislocation of each group were removed and homogenized, and

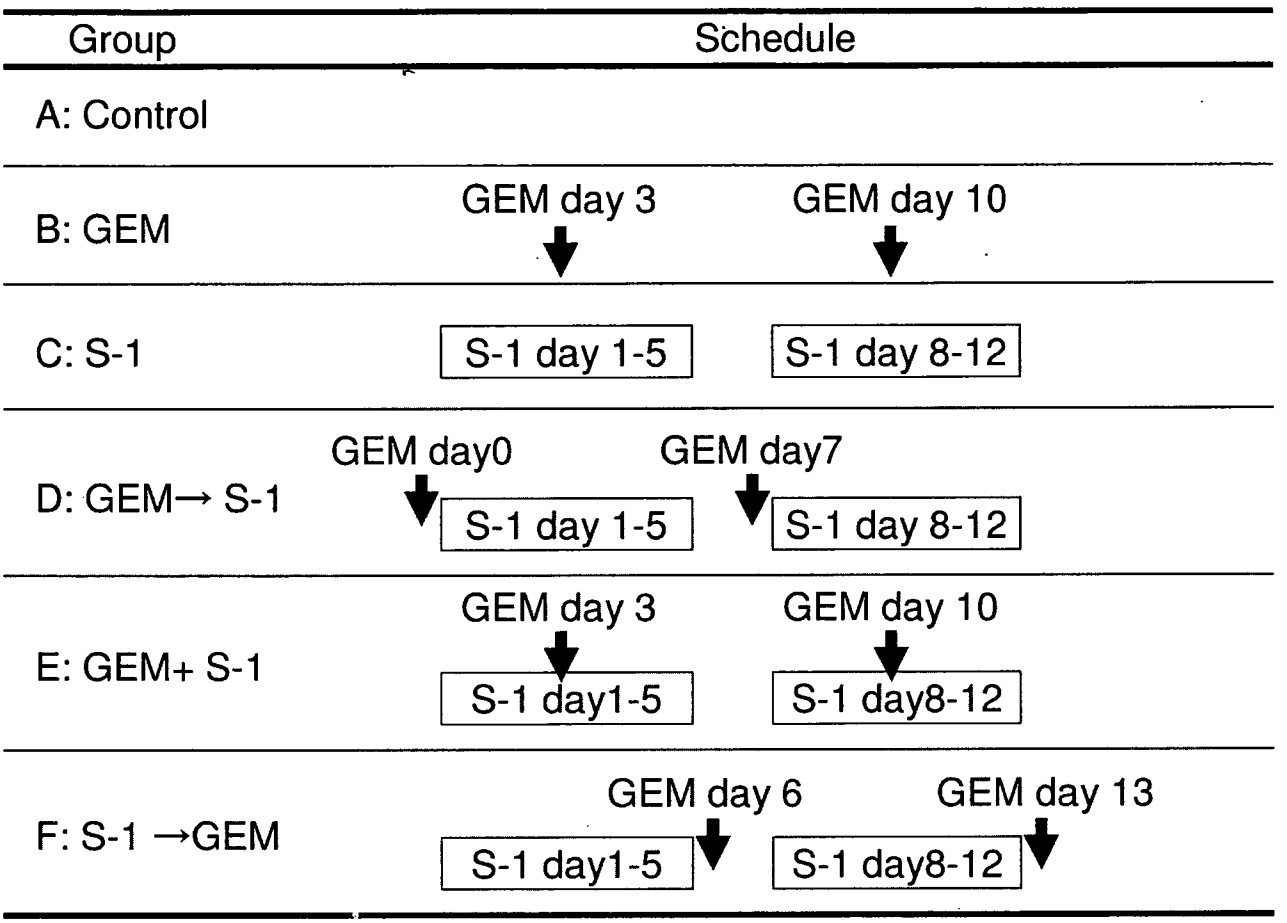

Figure 1. *Treatment schedule. MiaPaCa-2 cells ($5x10^6$ cells/100 μl phosphate-buffered saline) were injected subcutaneously into the right back of BALB/c mice. The mice were divided into 6 groups of 5 mice according to tumor volume. Gemcitabine (240 mg/kg) was administered by intraperitoneal injection and S-1 (10 mg/kg) by oral gavage. GEM: gemcitabine.*

quantitative gene expression of hENT1 mRNA in tumors was analyzed using LightCycler. The remaining three mice of each group were given a single intraperitoneal injection of [^{3}H] gemcitabine (240 mg/kg). Thirty minutes after gemcitabine injection, mice were killed by cervical dislocation, tumors were removed, weighed, and homogenized in Lumasolve solubilizer (LUMAC*LSC, Landgraaf, Netherlands). Gemcitabine concentrations in tumors were analyzed by quantitation of radioactivity as described elsewhere (27). The [^{3}H] gemcitabine uptakes were normalized by tumor weight.

Antitumor experiments in vivo. Mice bearing distinct tumors were randomly divided into the following six groups of 5 mice each (Figure 1): (A) no treatment; (B) weekly intraperitoneal injections of gemcitabine as a single agent (day 3, 10); (C) daily oral administrations of S-1 as a single agent for 5 consecutive days a week (day 1 to 5, day 8 to 12); (D) sequential combination treatment with gemcitabine (day 0, 7) prior to S-1 (day 1 to 5, day 8 to 12); (E) coadministrations of gemcitabine (day 3, 10) and S-1 (day 1 to 5, day 8 to 12); (F) sequential combination treatment with S-1 (day 1 to 5, day 8 to 12) prior to gemcitabine (day 6, 13). The animals were monitored for activity, physical condition, determination of body weight, and measurement of tumor volume [1/2 x (the major axis) x (the minor axis)^2] every other day. Statistical analysis of the data for the comparison of different groups was carried out using tumor volumes on day 18.

Statistical analysis. Statistical analysis was performed using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA, USA). Data were expressed as the average of experiments and represented as mean±SD. Differences between groups were examined for statistical significance using ANOVA. In all analyses, values of $p<0.05$ were considered statistically significant.

Results

hENT1 mRNA expression in pancreatic cancer xenograft tumors. The effect of S-1 on the hENT1 mRNA expression in xenograft tumors of MiaPaCa-2 was examined using quantitative RT-PCR (Figure 2). After the daily oral administrations of S-1 for 5 consecutive days, the tumors in

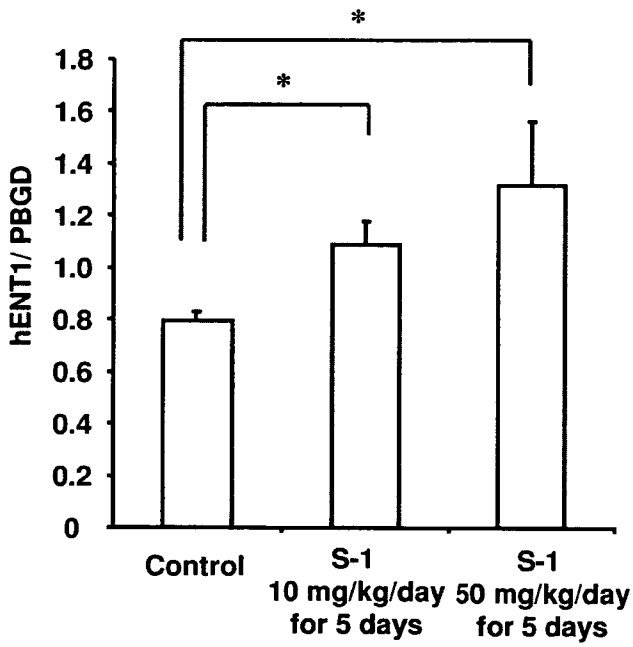

Figure 2. *Quantification of hENT1 mRNA expression in MiaPaCa-2 xenograft tumors treated with or without S-1. To quantify the data, hENT1 mRNA levels were normalized by PBGD mRNA levels. Results represent mean±SD (each group, n=3). The tumors in S-1 treated mice showed increased hENT1 levels. *p<0.05.*

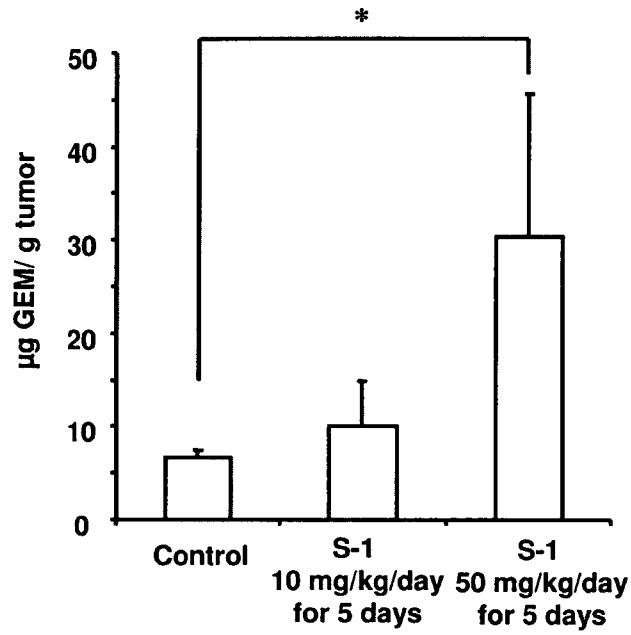

Figure 3. *Uptake of [^{3}H] gemcitabine after pretreatment with S-1 in pancreatic cancer xenograft tumors. The [^{3}H] gemcitabine uptakes were normalized by tumor weight. Results represent mean±SD (each group, n=3). The tumoral gemcitabine uptakes in S-1 treatment group at the dose of 50 mg/kg/day were significantly higher than the uptakes in control group. * p<0.05.*

S-1-treated mice showed increased hENT1 levels. The tumoral hENT1 expression in the S-1 treatment groups was significantly up-regulated compared with those in the control group.

Uptake of [^{3}H] gemcitabine after pretreatment with S-1 in pancreatic cancer xenograft tumors. The uptakes of gemcitabine were quantified using a [^{3}H] gemcitabine uptake assay (Figure 3). Although the S-1 treatment group at the dose of 10 mg/kg/day tended to show increased [^{3}H] gemcitabine uptake level, it was not significantly higher than that of the control group. However, the tumoral gemcitabine uptakes in S-1 treatment group at the dose of 50 mg/kg/day were significantly higher than the uptakes in the control group.

The schedule-dependent antitumor effect of gemcitabine and S-1 on human pancreatic tumor xenografts. The nude mice with the subcutaneous tumor were divided into six groups and treated as described in "Materials and Methods" and the antitumor effects were evaluated with the tumor volume at the day 18. The mean tumor volumes on day 18 were 4.2±1.5, 2.0±0.2, 2.3±1.0, 2.8±0.9, 2.1±0.7 and 1.0±0.3 cm^3 in Groups A, B, C, D, E and F, respectively (Figures 4 and 5). Tumor volumes of each treatment group were significantly smaller than those of the control group except for group D (GEM→S-1). Although there was not difference in tumor volumes among treatment groups B, C, D and E, statistically significant tumor growth inhibition was observed in Group F (S-1→GEM) compared with all the other treatment groups ($p<0.05$). Although the mice which were given S-1 first or simultaneously with gemcitabine showed stronger weight loss compared with other groups during and after the treatment, no mice died from the side-effects of the drugs or any other reasons up to the end of the study (day 18) (Figure 6).

Discussion

Although the systemic administration of gemcitabine is currently considered the standard first-line treatment for patients with advanced pancreatic cancer, single-agent gemcitabine has provided limited benefit, with objective response rates of less than 15% and a median survival of less than 6 months (28-30). Owing to the activity of gemcitabine, a variety of studies have now assessed its activity in combination with other agents. These studies have shown varying degrees of success, with no combination showing clear evidence of significantly superior activity (28). Combination of anticancer agents

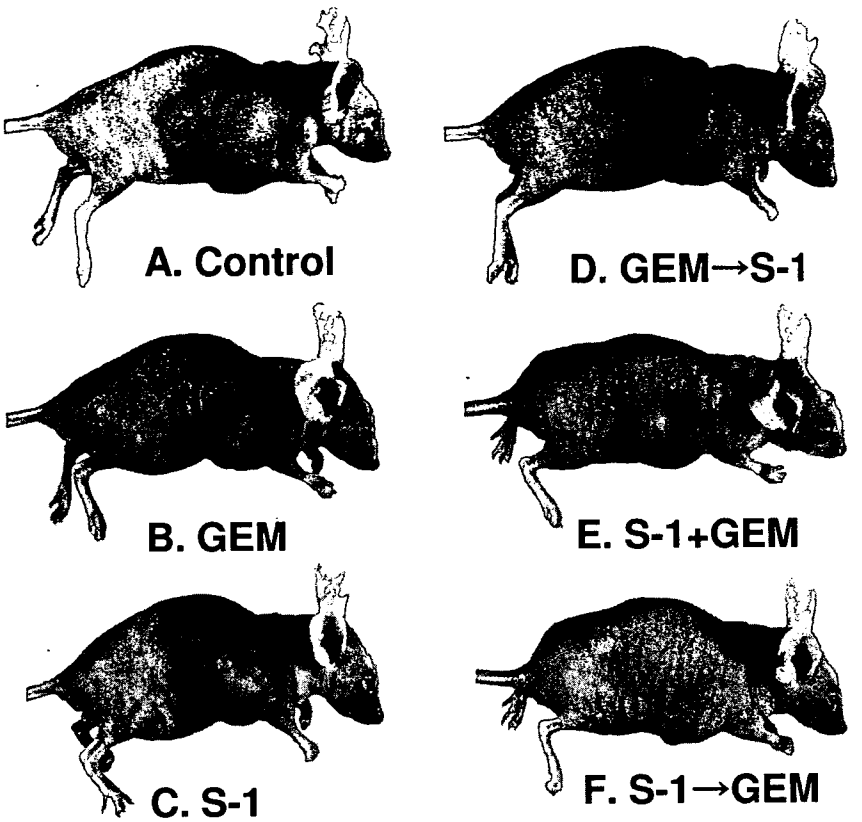

Figure 4. *BALB/c mice with subcutaneous human pancreatic tumor after treatment.*

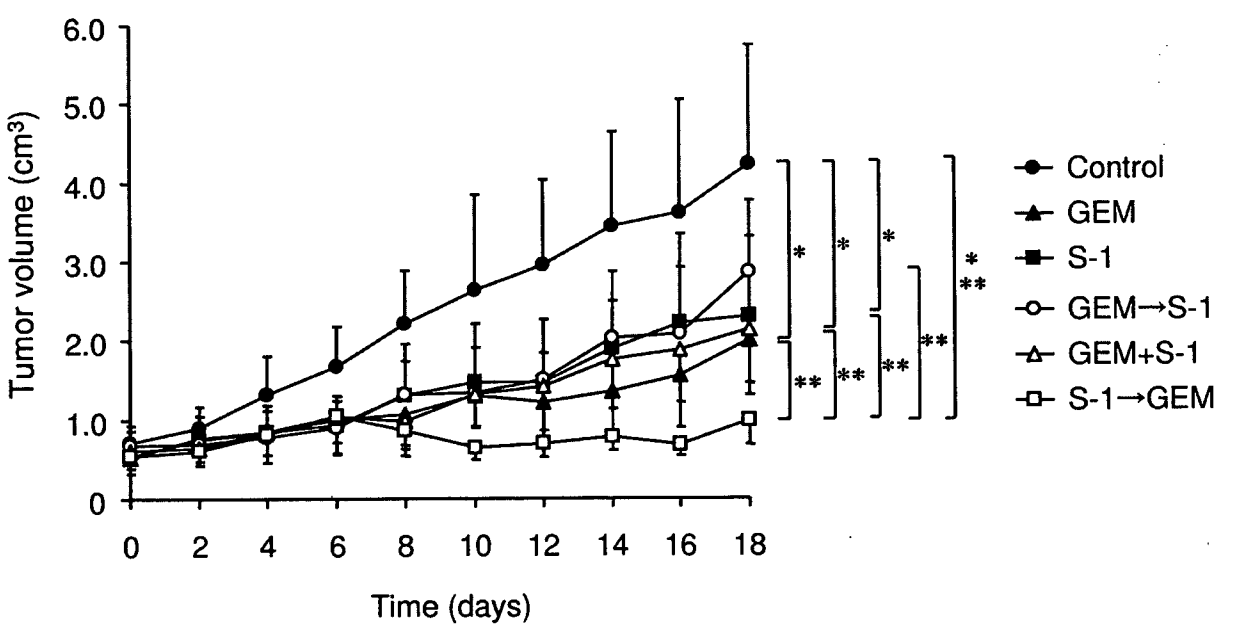

Figure 5. *Antitumor effect of the combination of gemcitabine with S-1 in vivo. Six different groups of the therapeutic experiments were carried out (Figure 1). Statistical analysis for the comparison of different groups was performed using tumor volumes on day 18. Results represent mean±SD (each group, n=5). Tumor volumes of each treatment group were significantly smaller than those of the control group except for group D (*p<0.05). Statistically significant growth inhibition was observed in Group F compared to all other treatment groups (**p<0.05). *, **p<0.05.*

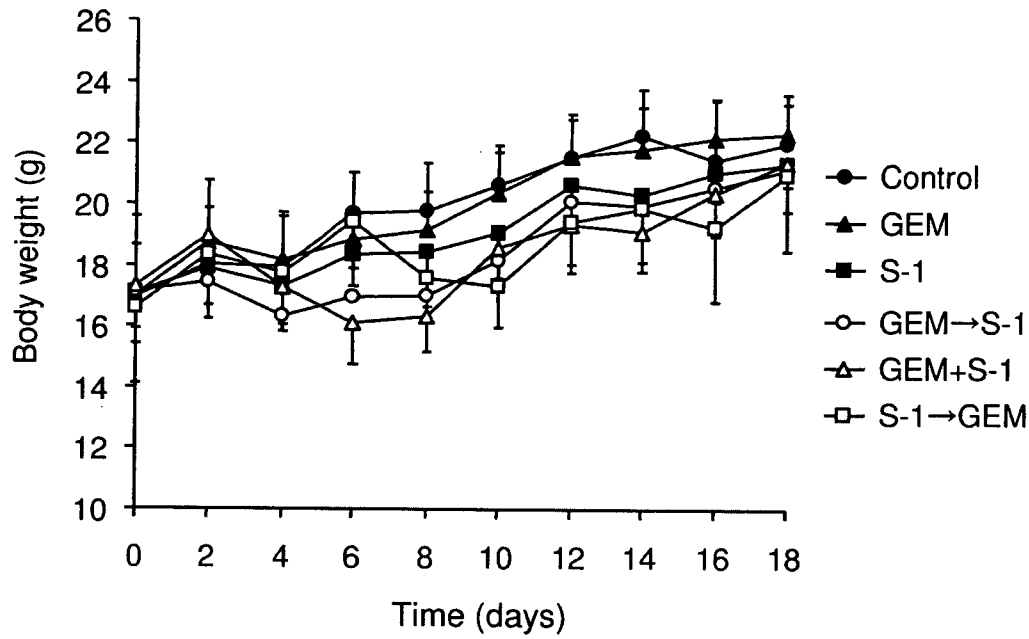

Figure 6. *Body weight curves of the mice. The corresponding tumor growth curves are shown in Figure 3. Results represent mean±SD (each group, n=5). There was no statistically significant difference between groups.*

has become a highly important modality for the treatment of cancer. Furthermore, combination chemotherapy is influenced by the employment of optimal scheduling (31). Gemcitabine is considered to be attractive for combination chemotherapy due to its mild toxicity profile at an active dose (16, 17).

However, in one study, the combination of 5-FU with gemcitabine did not improve the median survival of patients with advanced pancreatic carcinoma compared to single-agent gemcitabine (30). On the other hand, a novel oral derivative of 5-FU, S-1, was reported to have favorable results in pancreatic cancer patients (15), and a Phase II trial of S-1 combined with gemcitabine demonstrated promising efficacy with objective response rates of 48% and a median survival of 12.5 months (21). Therefore, we evaluated the effect of gemcitabine/S-1 combination therapy on human pancreatic cancer especially in terms of the schedule of the two drugs. Our study shows that the sequence can be an important factor in the antitumor activity of the combination of gemcitabine and S-1, and that hENT1 might play an important role in this effect.

The sequence-dependent effects of the combination of TS inhibitors and gemcitabine on human pancreatic cancer cells were reported to be seen with maximum effect when the TS inhibitors preceded gemcitabine *in vitro* (7). Moreover, this effect was not associated with basal hENT1 levels but with a significant increase in hENT1 levels caused by the TS inhibitors (7). Therefore, for *in vivo* experiments, we decided to use the cell lines which showed the highest rate of 5-FU induced increase in hENT1 mRNA (8).

We first examined the effects of S-1 on expression of hENT1 mRNA and gemcitabine cellular uptake in pancreatic cancer xenografts. 5-FU, known as a TS inhibitor, blocks the formation of 2'-deoxythymidine-5'-monophosphate (dTMP) and depletes intracellular nucleoside pools so that proliferating cells then depend on salvage of preformed nucleosides from extracellular fluid. 5-FU also up-regulates the amount of cell surface hENT1 as confirmed by flow cytometric analysis (7). In this study, we demonstrated that treatment with S-1 resulted in hENT1 up-regulation at the mRNA level and increased tumoral gemcitabine uptake.

To clarify the optimal schedule of treatment, we investigated the effect of six different schedules of treatment of S-1 and/or gemcitabine on a xenograft model of MiaPaCa-2 tumors. We did not detect any significant differences among the monotherapy groups and combination groups except for the S-1 followed by gemcitabine group. However, significant tumor growth inhibition was observed in mice treated with S-1 followed by gemcitabine compared with either untreated mice or the other treatment groups. Moreover, the synergistic tumor growth inhibitory effect was observed only in the S-1 followed by gemcitabine group and not in the other

combination groups. These results suggest that the schedule in which the gemcitabine is administered after S-1 could be the optimal combination of these two agents in the treatment of pancreatic cancer. The up-regulation of hENT1 induced by S-1 may play an important role in the enhanced growth inhibitory effect of gemcitabine.

Moreover, only the gemcitabine followed by S-1 treatment group did not show any efficacy compared with the control group. Previous reports showed that treatment of gemcitabine increased hENT1 expression and reduced 5-FU sensitivity in human pancreatic cancer cell lines (7). Therefore, the pretreatment with gemcitabine might reduce the effects of 5-FU due to the increasing supply of nucleosides and nucleobases *via* the salvage pathway. Indeed, the expression level of basal hENT1 was inversely associated with 5-FU sensitivity in human pancreatic cancer cell lines (8). These data suggested that the lack of benefit with sequences other than that of S-1 followed by gemcitabine *in vivo* might be due to gemcitabine-induced up-regulation of hENT1 and subsequent reduced 5-FU sensitivity.

It has been reported that mouse ENT1 (mENT1) mRNA was highly expressed in the heart, spleen, lung, liver and testes, and that lower levels of expression were detected in the brain and kidney (32). Therefore, enhanced gemcitabine effects induced by S-1 may also aggravate gemcitabine-induced side-effects. However, in this study, neither severe side-effects nor mortality were observed, although the mice treated with S-1 first or simultaneously with gemcitabine showed greater weight loss than other groups.

In conclusion, our results showed that the administration of S-1 followed by gemcitabine provides greater inhibitory effects than other gemcitabine/S-1 schedules in the treatment of pancreatic cancer.

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Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer

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Pancreatic cancer is the most lethal of all solid tumors partially because of its chemoresistance. Although gemcitabine is widely used as a first selected agent for the treatment of this disease despite low response rate, molecular mechanisms of gemcitabine resistance in pancreatic cancer still remain obscure. The aim of this study is to elucidate the mechanisms of gemcitabine resistance. The 81-fold gemcitabine resistant variant MiaPaCa2-RG was selected from pancreatic cancer cell line MiaPaCa2. By microarray analysis between MiaPaCa2 and MiaPaCa2-RG, 43 genes (0.04%) were altered expression of more than 2-fold. The most upregulated gene in MiaPaCa2-RG was ribonucleotide reductase M1 subunit (RRM1) with 4.5-fold up-regulation. Transfection with RRM1-specific RNAi suppressed more than 90% of RRM1 mRNA and protein expression. After RRM1-specific RNAi transfection, gemcitabine chemoresistance of MiaPaCa2-RG was reduced to the same level of MiaPaCa2. The 18 recurrent pancreatic cancer patients treated by gemcitabine were divided into 2 groups by RRM1 levels. There was a significant association between gemcitabine response and RRM1 expression ($p = 0.018$). Patients with high RRM1 levels had poor survival after gemcitabine treatment than those with low RRM1 levels ($p = 0.016$). RRM1 should be a key molecule in gemcitabine resistance in human pancreatic cancer through both *in vitro* and clinical models. RRM1 may have the potential as predictor and modulator of gemcitabine treatment.
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Key words: gemcitabine; pancreatic cancer; drug resistance; RRM1; microarray

Pancreatic cancer remains one of the most malignant cancers. Although surgery is the only curative treatment currently available, over 80% of patients have advanced regional disease or distant metastasis at the time of diagnosis and less than 20% of the patients are candidates for resection.[1] Therefore, chemotherapy, radiation or a combination of these therapies most commonly plays an important role in pancreatic cancer treatment. They have not had a significant impact on survival rates in recent decades, however, despite many clinical trials.[1]

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC, Gemzar) has been recognized as the standard first-line chemotherapeutic agent used in patients with pancreatic cancer, since it was shown to have some meaningful impact on either survival or disease-related symptoms when compared with 5-fluorouracil (5-FU) in randomized trials.[2] However, not more than 25% patients with pancreatic cancer will benefit from gemcitabine, a proportion that is slightly less than in patients with other cancers.[2] Although gemcitabine in combination with other various cytotoxic agents is being investigated, no randomized phase III trial has yet established any survival benefit for combination therapy when compared with gemcitabine alone.[1,3] The major cause of this relative treatment failure is thought to be tumor cell resistance to chemotherapy, whether it is inherent or acquired.[4]

A variety of attempts have recently been undertaken *in vitro* to detect the molecular markers of gemcitabine resistance. Alterations involved in cell cycle regulation, proliferation or apoptosis, such as mutated *p53*,[5] Bcl-xl,[6] c-*Src*,[7] focal adhesion kinase[8] and BNIP3,[9] have been described in a variety of cancers including pancreatic cancer. Nucleotide transporters were also described as molecules related to the intracellular transport of extracellular gemcitabine from outside.[10,11] The ribonucleotide reductase M1 subunit (RRM1),[12,13] ribonucleotide reductase M2 subunit (RRM2),[14,15] deoxycytidine kinase (dCK)[16] and cytidine deaminase (CDA)[17] are supposed to play a role in gemcitabine resistance of the variety of cancer as metabolic enzymes of the drug. However, they remain still controversial because of the lack of direct evidence based on either *in vitro* gene transfer model systems or clinical data from patients with pancreatic cancer.

Variant cells with characteristics resistant to chemotherapeutic agents have widely contributed to the investigation of molecular mechanisms in chemoresistance.[7,12,13,16,18,19] These chemoresistant variants are traditionally established by continuous drug exposure and gradually increased drug concentration. Although drug resistance can occur at many levels, including increased drug efflux, drug inactivation, alterations in drug target, processing of drug-induced damage and evasion of apoptosis,[20] the advent of recently established analytical technologies such as microarray and protein array systems has opened up feasible opportunities to identify molecules involved in drug resistance. Indeed, microarray analysis has become a key tool for characterizing gene expression in a variety of experimental systems with chemoresistant variants and has succeeded in identifying the molecules associated with gemcitabine resistance using an oligonucleotide microarray system *in vitro* and *in vivo*.[12,13,21]

In this study, we developed gemcitabine-resistant cells from the human pancreatic cancer cell lines and attempted to identify novel genes involved in gemcitabine chemoresistance using an oligonucleotide microarray system covering 30,000 human oligonucleotides. Furthermore, the detected candidate gene was also revealed to be responsible for gemcitabine resistance by an RNAi assay and by clinical analysis of the patients treated with gemcitabine.

Material and methods

Pancreatic cancer cell lines and selection of gemcitabine resistant cells

Five types of human pancreatic carcinoma cell lines were used in the present study. MiaPaCa-2 and PSN1 cell lines were obtained from the Japanese Collection of Research Bioresources (JCRB, Tokyo, Japan). The BxPC3 and Panc1 cell lines were obtained from the American Type Culture Collection (ATCC,

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Rockville, MD). The PCI6 cell line was a gift from Dr. H. Ishikawa (Hokkaido University, Sapporo, Japan). All cell lines were cultured at 37°C under 5% CO_2 in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (Hyclone Laboratories, Inc., Rockville, MD) and 100 units/ml each of penicillin and streptomycin. Relative gemcitabine-sensitive cell lines, BxPC3, PSN1 and MiaPaCa2 were used for the establishment of chemoresistant variants. Gemcitabine-resistant cells were generated by exposure to gradually increasing concentrations of the drug for 2 months as described previously.[12,22] The starting concentration was 1 ng/ml gemcitabine. When cells adapted to the drug, the gemcitabine concentration was increased. The final concentrations were 10 ng/ml gemcitabine for PSN1 and 20 ng/ml gemcitabine for BxPC3 and MiaPaCa-2.

Reagents

Gemcitabine was kindly provided by Eli Lilly Pharmaceuticals (Indianapolis, IN). 5-Fluorouracil (5-FU) was purchased from Sigma Chemical Co. Gemcitabine and 5-FU were dissolved in distilled water and applied to cells at a concentration of less than 0.1% of the medium volume.

Cytotoxicity assay

Cell growth was assessed by the 3-(4-, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co.) method.[23] Briefly, 3×10^4 cells were seeded to a 96-well plate in 100 µl of medium and left overnight to adhere. Several concentrations of the test drugs in 100 µl volumes were added, and the cells were incubated for 48 hr. After treatment, 10 µl of MTT solution (5 mg/ml) was added to each well and incubated for another 4 hr at 37°C. Then, 100 µl of acid-isopropanol was added, and after 24 hr at 4°C, reduced MTT was measured spectrophotomechanically in a dual beam microtiter plate reader at 570 nm with a 650 nm reference. Resulting absorbencies were converted to percent survival by comparing treated with untreated (100% survival) cells. 50% inhibitory concentrations (IC_{50}s) are defined as the concentrations of drug that result in 50% cell survival when compared with untreated cells.

Growth curve

Cells (1×10^4) were seeded to a 24-well plate in 1 ml of medium and left overnight to adhere. The medium was replaced daily with 1 ml of fresh medium with or without gemcitabine at the dose of parental IC_{50}. Cell numbers were counted with an automatic cell counter (Celltec MEK-5103, Nihon Kohden, Tokyo, Japan) after being treated with trypsin.

Animals and in vivo *antitumor experiments*

Four-week-old female BALB/c nu/nu mice were purchased from Japan Clea (Tokyo, Japan) and maintained in specific pathogen-free conditions. Human pancreatic tumor xenografts were prepared by subcutaneous implantation (5×10^6 cells; total volume 100 µl) of MiaPaCa2 and MiaPaCa2-RG, resistant variant established from MiaPaCa2, into the right back of 10 nude mice each. The animals were monitored for activity, physical condition, determination of body weight and measurement of tumor volume [1/2 × (the major axis) × (the minor axis)^2] every other day. When the tumors reached a volume between 100 and 200 mm^3, mice were divided into the following 4 groups of 5 mice each: parental cell with no treatment, parental cell with weekly intraperitoneal injections of gemcitabine, resistant cell with no treatment, resistant cell with weekly intraperitoneal injections of gemcitabine. Gemcitabine was injected weekly into the peritoneal cavity at the dose of 240 mg/kg as described.[24]

[^{3}H] gemcitabine cellular uptake assay

Cells were seeded to a flat-bottomed 24-well microplate (1×10^4/well) and incubated for 24 hr. The medium was replaced by 1 ml of fresh medium by an additional 48 hr of culture. The cells were then exposed to [^{3}H] gemcitabine (Moravek Biochemicals, Inc. Brea, CA) at a concentration of 23.9 ng/ml (1.0 µCi/ml). After 1-hr exposure, the cells were washed 3 times in 1 ml of ice cold phosphate-buffered saline (PBS). The cells were then dissolved in 0.5 ml of 0.5% Triton X-100, and 0.4 ml aliquots were sampled for radioactivity counting. Aliquots of 20 µl were also sampled for protein determination. The uptake level of [^{3}H] gemcitabine was expressed as radioactivity levels divided by protein concentrations measured by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

TABLE I – IC_{50} OF GEMCITABINE (GEM) AND 5-FLUOROURACIL (5-FU) IN GEMCITABINE-SELECTED PANCREATIC CANCER CELL LINES

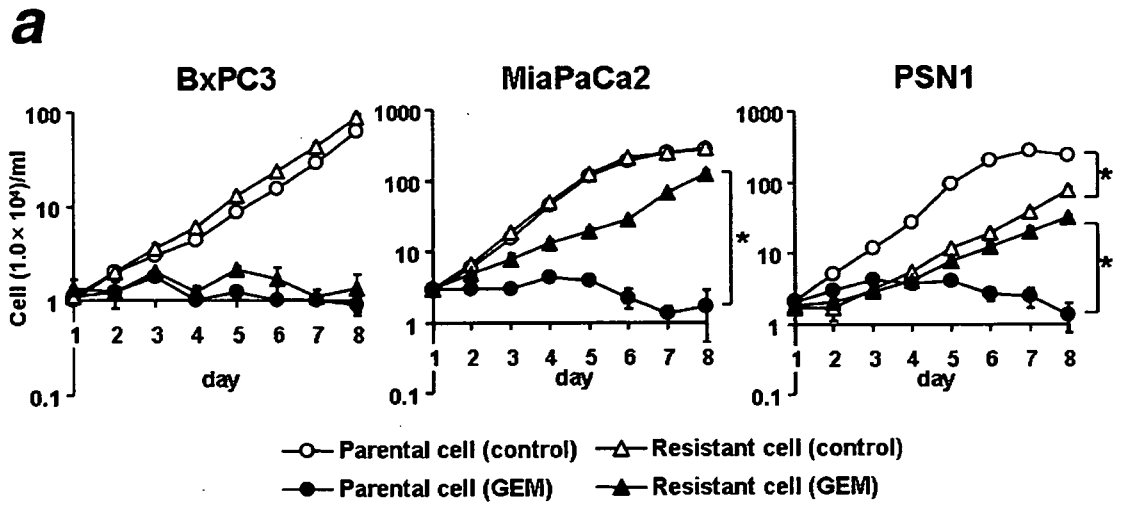
Cell lines	Drug	IC_{50} (mean ± SD)		Fold resistance
		Parental cell	Resistant cell	
BxPC3	GEM (ng/ml)	50.5 ± 7.1	556.6 ± 76	11-fold
	5-FU (µg/ml)	0.6 ± 0.2	37.5 ± 4.1	60-fold
MiaPaCa2	GEM (ng/ml)	44 ± 5.3	3592.1 ± 170	81-fold
	5-FU (µg/ml)	2.4 ± 0.3	3.07 ± 0.14	1.3-fold
PSN1	GEM (ng/ml)	3.4 ± 0.3	3392 ± 44	986-fold
	5-FU (µg/ml)	1.4 ± 0.1	45 ± 2.6	32-fold

Oligonucleotide microarray

RNA extraction was carried out with TRIzol reagent (Invitrogen, Carlsbad, CA) using a single-step method,[25] and RNA quality was checked with an RNA 6000 Nano LabChip kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's protocols. An oligo-microarray covering 30,000 human oligonucleotides (AceGene human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Ltd., Yokohama, Japan) was used in this study.[26] Sample preparation, hybridization and wash were carried out according to the manufacturer's protocols (http://www.dna-chip.co.jp/thesis/AceGeneProtocol.pdf). A sample and the reference were labeled with Cy5-dUTP and Cy3-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ), respectively, mixed, and hybridized on a microarray. The hybridized array was scanned using ScanArray 4000 (GSI Lumonics) at wavelengths corresponding to each probe's unique fluorescence (635 and 532 nm for Cy5 and Cy3, respectively). The signal intensity of each spot (16 bit tiff image) was converted into text format by DNASISArray software (Hitachi software Inc., Tokyo, Japan). Data processing was performed through background subtraction using the average blank spot intensity in each block. If the signal was higher than the background and the signal levels of Cy3 and Cy5 were higher than 1,000, these data were used for further analysis. At this stage, 10,517 genes remained. The Cy3/Cy5 ratio values of each spot were log-transformed and normalized so that the median Cy3/Cy5 ratio of whole genes was 1.0.[27]

Reverse transcription-polymerase chain reaction

RNA extraction was carried out with TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was generated with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), as described previously.[25] In this assay, porphobilinogen deaminase (PBGD) mRNA was used as an internal control.[28] PCR was performed in a 25-µl reaction mixture containing 2 µl of cDNA template, 1× Perkin-Elmer PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 0.2 µM each primer and 1 U of Taq DNA polymerase (AmpliTaq Gold, Roche Molecular System, Inc.). The primers for PBGD were synthesized as described previously.[28] The PCR primers used for the detection were as follows: dCK (forward primer, 5′-TGCAGGGAAGTCAACATT-3′; reverse primer, 5′-TCCCACCATTTTTCTGAG-3′), CTP synthetase (forward primer, 5′-CTCATATCACAGATGCAATC-3′; reverse primer, 5′-GATCATATCTGTCAGCCATCTC-3′), CDA (forward primer, 5′-GGAGGCCAAGAAGTCAG-3′; reverse primer, 5′-GACGGCCTTCTGGATAG-3′), DCTD (forward primer, 5′-GTGCAGTGATGACGTGTGTTGC-3′; reverse primer, 5′-CATGTAGATTCCATGTGAC-3′), RRM1 (forward primer, 5′-GAAGACTGGGATGTATTATTTAAG-3′; reverse primer, 5′-CAGAATAACCTATAGGAC-3′), RRM2 (for-



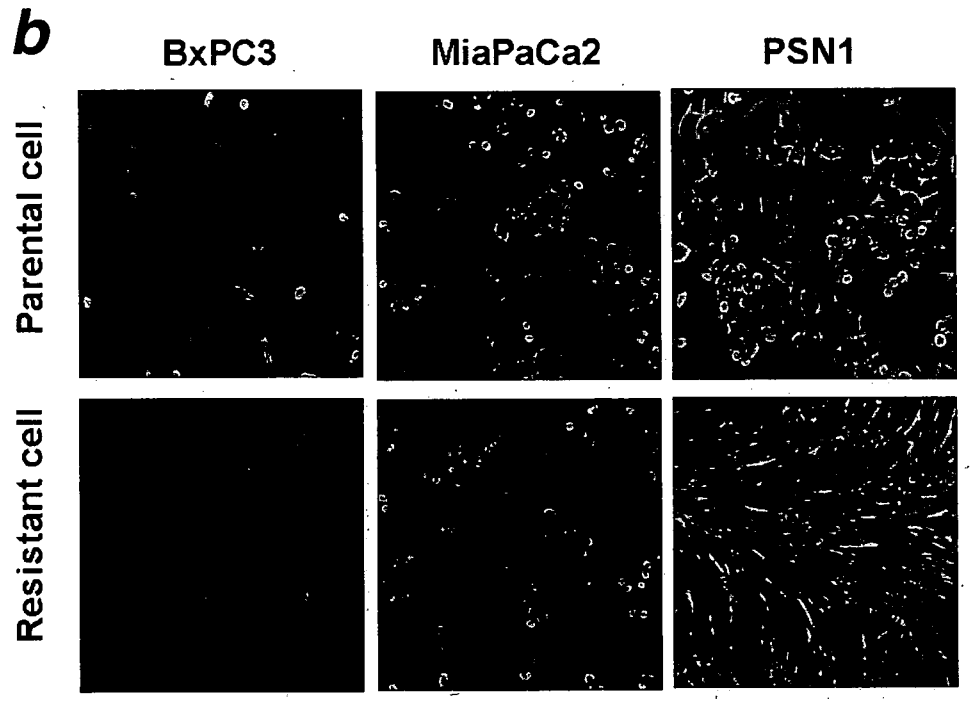


FIGURE 1 – (*a*) Growth curves of pancreatic cancer cell lines. In the absence of gemcitabine (open circle, open triangle) and in continuous exposure to gemcitabine at the dose of parental IC_{50}s (closed circle and closed triangle). Points, mean; bars, SD ($n = 3$). *$p < 0.01$. (*b*) Morphology of pancreatic cancer cell lines, BxPC3, MiaPaCa2, PSN1 and gemcitabine-resistant variants, BxPC3-RG, MiaPaCa2-RG, PSN1-RG. (Original magnification, ×40).

ward primer, 5′-ATGAAAACTTGGTGGAGCGATT-3′; reverse primer, 5′-TGGCAATTTGGAAGCCATAGA-3′), p53R2 (forward primer, 5′-CCAGTTGGCCTCATTGGAAT-3′; reverse primer, 5′-TAGAGTTTTAAAACGAGAGG-3′), ENT1 (forward primer, 5′-GCTTGAAGGACCCGGGGAGC-3′; reverse primer, 5′-TGGAGAAGGCAAAGGCAGCCA-3′). PCR was performed with cycling conditions of 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 62°C (RRM1: 57°C) for 30 sec and extension at 72°C for 60 sec, and the products were run on 2% agarose gels and visualized by ethidium bromide staining. A quantitative gene expression assay was performed using LightCycler (Idaho Technology, Salt Lake City, UT), as described previously.[29] PCR was performed with cycling conditions of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 62°C (RRM1: 57°C) for 10 sec and extension at 72°C for 20 sec. Quantification data from each sample were analyzed using the LightCycler analysis software (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer. Relative gene expression levels are expressed as quantified gene expression divided by quantified PBGD levels.

Western blotting

Cells grown to subconfluence in 90-mm dishes were lysed in protease inhibitor (1 mM PMSF, 40 μM leupeptin) containing PBS. After sonication, aliquots containing 50 μg of total protein were size-fractionated by SDS-PAGE (5–20% gradient gels), and the proteins were transferred to polyvinylidine difluoride membranes (Immobilon, Millipore, Bedford, MA) as described previously.[25] The membranes were blocked with 5% skim milk and

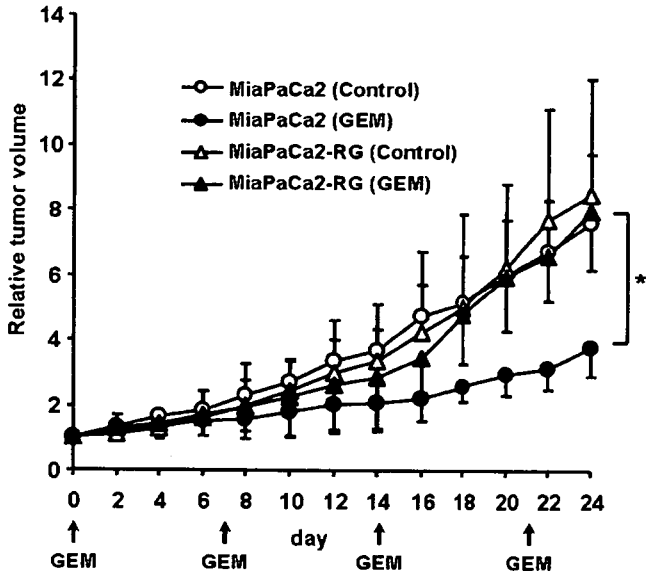


FIGURE 2 – *In vivo* gemcitabine sensitivity of MiaPaCa2 and MiaPaCa2-RG in subcutaneous xenograft model of nude mice. Gemcitabine was injected weekly into the peritoneal cavity at the dose of 240 mg/kg (closed circle and closed triangle) and control (open circle, open triangle). Points, mean; bars, SD ($n = 5$). *$p < 0.01$.

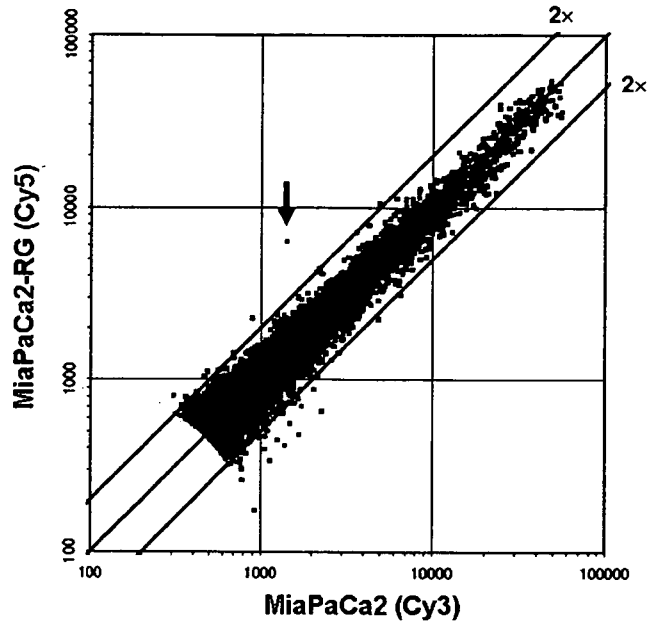


FIGURE 3 – Representative scatterplots showing hybridizations of cDNA from MiaPaCa2 cells labeled Cy3 and MiaPaCa2-RG cells labeled Cy5. The lines show 2-fold difference expression in both channels. RRM1; arrow.

incubated for 1 hr at room temperature with mouse monoclonal anti-human RRM1 (Chemicon international, Inc., Temecula, CA) or rabbit polyclonal anti-human actin (Sigma). After 3 washings with 0.1% Tween 20 in TBS, the membranes were incubated for 30 min at room temperature with the horseradish peroxidase-conjugated secondary antibody. After further 5 washings peroxidase was detected with an enhanced chemiluminescence system from Amersham (Arlington Heights, IL).

RNAi treatment

RRM1 and control RNAis were purchased from Invitrogen (Stealth RNAi; Invitrogen, Carlsbad, CA). The RRM1-specific RNAi designed by BLOCK-iT RNAi Designer (Invitrogen, Carlsbad, CA) was as follows: Sense 5′-GGAUAUUGUUCUGGCCAAUAAAGAU-3′; Anti-sense 5′-AUCUUUAUUGGCCAGAACAAUAUCC-3′. A stealth RNAi negative control with medium GC duplex was used as a control. RNAis were dissolved in DEPC-treated water to make a 20 μM working stock. One day before transfection, 2×10^5 cells were plated into 35 mm, 6-well trays and allowed to adhere. Transfection was performed using Lipofectamine 2000 transfection regent (Invitrogen, Carlsbad, CA) following Invitrogen's protocols. The ability of the RNAi molecules to knock down RRM1 expression was analyzed by mRNA and protein detection, and the final dilution volume of RNAi was 50 pmol in 500 μl OptiMEM medium per well.

Patients and tissue samples

Eighteen recurrent pancreatic cancer patients in Osaka University Hospital were recruited. All patients had undertaken curative resection at Osaka University Hospital between September 1999 and February 2004 and were followed-up without any adjuvant treatment until recurrence. The tumor tissues had been collected and stored at −80°C until use under informed written consent. Each tumor was confirmed histopathologically to be advanced stage cancer. All patients had a measurable recurrent lesion and were treated with only gemcitabine after recurrence. Response to gemcitabine was defined as follows: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). This classification was based on New Guidelines to Evaluate the Response to Treatment in Solid Tumors (RECIST guidelines).[30] Patients were divided into 2 groups based on the chemotherapeutic response. Responders were defined as CR, PR and SD. Nonresponders were PD. A total of 18 tumor samples resected at the primary curative operation were analyzed to determine RRM1 mRNA expression levels. Total RNA was isolated from the homogenate tumor samples using TRIzol method[27] for quantitative reverse transcription-polymerase chain reaction (RT-PCR) using same conditions as already described. Total RNA of MiaPaCa2-RG was used for analytical curve.

Statistical analysis

Statistical analyses were performed using the SPSS 11.5J software (SPSS Inc., Chicago, IL). All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t* test. In the clinical study, associations between the candidate molecule expression and gemcitabine response were assessed by Fisher's exact test. Overall, survival probabilities were estimated using the Kaplan-Meier method, and the log-rank test was used to determine the level of significance between the survival curves. A *p* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Establishment of gemcitabine-resistant pancreatic cancer cell lines

Three types of pancreatic cancer cell lines, BxPC3, PSN1 and MiaPaCa2, were cultured in the medium containing gemcitabine for 2 months. After selection, we established 3 variant cells resistant to gemcitabine with different degrees with the MTT assay (Table I). The selected cell lines were called BxPC3-RG, MiaPaCa2-RG or PSN1-RG, based on the names of their parental cell line. On the basis of the IC_{50} measurement, BxPC3-RG, MiaPaCa2-RG and PSN1-RG were 11-fold, 81-fold and 986-fold more resistant than parental cells to the cytotoxic effects of gemcitabine, respectively. BxPC3-RG and PSN1-RG were also cross-resistant to 5-FU, although MiaPaCa2-RG represented no significant

TABLE II – GENES UP- AND DOWNREGULATED BY MORE THAN 2-FOLD IN MiaPaCa2-RG WITH MIAPACA2 AS A REFERENCE

	Fold	Gene name	Symbol	Accession no.
Upregulated				
1	4.46	Ribonucleotide reductase m1 polypeptide	RRM1	NM_001033
2	2.63	ensembl genscan prediction		AL050329
3	2.29	kiaa0101 gene product	KIAA0101	NM_014736
4	2.27	Hypothetical protein	ATP5S	NM_015684
5	2.20	Inosine monophosphate dehydrogenase 1	IMPDH1	XM_004627
6	2.19	Hypothetical protein flj20558	FLJ20558	NM_017880
7	2.09	Suppression of tumorigenicity 7	ST7	NM_018412
8	2.07	Hypothetical protein xp_040263	LOC91732	XM_040263
9	2.06	Suppressor of g2 allele of skp1	SUGT1	NM_006704
10	2.02	Unknown (protein for image:3456579)	FUBP3	BC001325
11	2.00	ba196n14.4.1 (pro1085 protein, isoform 1)		AL354776
12	2.00	Hypothetical protein xp_039528	LOC91613	XM_039528
Downregulated				
1	5.32	ensembl genscan prediction		AC063943
2	3.50	Antiquitin	ALDH7A1	NM_001182
3	3.49	ensembl genscan prediction		AC068601
4	3.36	Adenylate cyclase 6, isoform b	ADCY6	NM_020983
5	3.31	ensembl genscan prediction		AC009294
6	3.01	Activator of s phase kinase	ASK	NM_006716
7	2.78	udp glycosyltransferase 2 family, polypeptide b4	UGT2B4	NM_021139
8	2.69	ensembl genscan prediction		AF131216
9	2.66	ensembl genscan prediction		AF277315
10	2.54	Unknown	C9orf10	AF055017
11	2.48	Potassium voltage-gated channel, shal-related subfamily, member 3	KCND3	NM_004980
12	2.34	ensembl genscan prediction		AC005034
13	2.29	ensembl genscan prediction		AL356751
14	2.26	ensembl genscan prediction		AL135978
15	2.23	ensembl genscan prediction		AC021883
16	2.21	Adaptor-related protein complex 2, mu 1 subunit	AP2M1	NM_004068
17	2.21	Phosphoserine phosphatase	PSPH	NM_004577
18	2.18	Transaldolase-related protein	TALDO1	AF010400
19	2.16	Hypothetical protein xp_016148	LOC95556	XM_016148
20	2.15	Transcription elongation factor a (sii), 1	TCEA1	NM_006756
21	2.14	Hepatitis a virus cellular receptor 1	HAVCR1	NM_012206
22	2.13	Protein phosphatase 1, regulatory (inhibitor) subunit 2	PPP1R2	NM_006241
23	2.12	Ring finger protein 22, isoform beta	TRIM3	NM_033278
24	2.11	Glycine cleavage system protein h	GCSH	NM_004483
25	2.07	Hypothetical protein nuf2r	CDCA1	BC008489
26	2.06	Dj1093g12.6 (a novel protein)	C20orf93	AL121751
27	2.05	Inosine-5′-monophosphate dehydrogenase	IMPDH2	J04208
28	2.02	Hypothetical protein xp_052919	LOC112547	XM_052919
29	2.02	ensembl genscan prediction		AL132801
30	2.01	ensembl genscan prediction		AC010553
31	2.00	Heme-regulated initiation factor 2-alpha kinase	HRI	NM_014413

cross-resistance (Table I). By the growth curve analysis, MiaPaCa2-RG and PSN1-RG showed significant resistant to gemcitabine, although BxPC3-RG did not show any resistance to gemcitabine. In the absence of gemcitabine, BxPC3-RG and MiaPaCa2-RG demonstrated almost the same growth curves when compared with parental cells, although PSN1-RG's growth rate was 10-fold slower than PSN1 (Fig. 1*a*). BxPC3-RG and MiaPaCa2-RG preserved the cell morphology of parental cells regardless of these chemoresistant alterations, and PSN1-RG showed significant difference in the cell morphology (Fig. 1*b*). MiaPaCa2-RG remained gemcitabine-resistant after 1 month culture in the medium without gemcitabine. Furthermore, MiaPaCa2-RG showed significant gemcitabine-resistance when compared with MiaPaCa2 in an *in vivo* xenograft model (Fig. 2). The level of [^{3}H] gemcitabine cellular uptake in MiaPaCa2-RG (25.0 ± 3.2 pg GEM/μg protein) is half of that in MiaPaCa2 (49.9 ± 5.8 pg GEM/μg protein). These data suggest that MiaPaCa2-RG should be the most suitable for identifying genetic alterations relating to gemcitabine resistance among the 3 types of gemcitabine-selected variants. We chose MiaPaCa2-RG for further analysis to identify molecules associated with gemcitabine resistance.

Microarray analysis

To investigate the candidate genes involved in gemcitabine resistance, oligo-microarray experiments were carried out with MiaPaCa2 and MiaPaCa2-RG cells. Out of the 30,000 spotted genes, 10,517 genes were used for further analysis (See Material and methods). Scatter plotting showed that 99.6% genes (10,474 genes out of 10,517 genes) had altered expressions of less than 2-fold, and 43 genes were up- or downregulated more than 2-fold in MiaPaCa2-RG cells when compared with MiaPaCa2 cells (Fig. 3). Among the 43 genes in which 12 upregulated genes and 31 downregulated genes were identified, the RRM1 was the most upregulated with 4.5-fold (Table II). This gene is the subunit of ribonucleotide reductase (RR) considered as an enzyme associated with gemcitabine metabolism.[4] This upregulation was validated by both quantitative RT-PCR and Western blotting (data not shown). Other subunits of RR, RRM2 and p53R2, and other enzymes involved in gemcitabine metabolism such as CDA, dCK, CTP synthetase and dCMP deaminase and nucleotide transporters were not chosen in the microarray analysis because of their low expression levels or failure to show any altered expression between MiaPaCa2 and MiaPaCa2-RG with quantitative RT-PCR. The func-

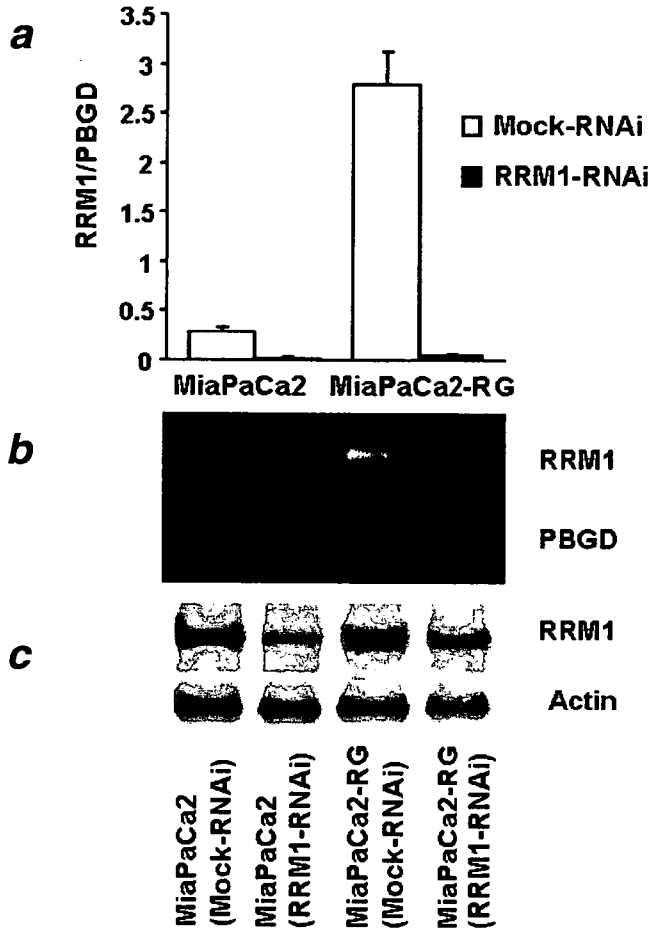


FIGURE 4 – (*a*) Quantitative RT-PCR, (*b*) RT-PCR and (*c*) Western blotting analyses of RRM1 expression after RRM1-specific RNAi transfection in MiaPaCa2 and MiaPaCa2-RG cells. Columns, mean; bars, SD (n = 3).

tions of other up- and downregulated genes were not considered to have any association with gemcitabine sensitivity. Therefore, we focused on the RRM1 gene for further functional analysis.

Chemosensitivity after RRM1-specific RNAi transfection

To verify that RRM1 should be involved in gemcitabine resistance, RNAi experiments were carried out on MiaPaCa2 and MiaPaCa2-RG. The ability of RRM1-specific RNAi to suppress RRM1 expression was confirmed by both RT-PCR (Figs. 4*a* and 4*b*) and Western blotting (Fig. 4*c*). After transfection with RRM1-specific RNAi, more than 90% suppression of RRM1 was observed (Fig. 4*a*). Other subunits of ribonucleotide reductase, RRM2 and p53R2, did not have any significant mRNA expression change. RRM1-specific RNAi transfection did not bring about any major effect on cell viability. After RRM1-specific RNAi transfection, the gemcitabine chemoresistance of MiaPaCa2-RG was significantly reduced to same level as that of MiaPaCa2, and gemcitabine response of MiaPaCa2 also became more sensitive (Fig. 5).

RRM1 expression and gemcitabine response in human pancreatic cancer cells and patients with pancreatic cancer

To investigate that the increased expression also should be involved in intrinsic resistance to gemcitabine, we examined the association between RRM1 mRNA expression levels and gemcitabine sensitivity of 5 human pancreatic cancer cell lines at first. RRM1 mRNA expression levels are significantly associated with gemcitabine sensitivity in 5 pancreatic cancer cell lines (Fig. 6), although increased expression of RRM1 was not likely correlated with the increase of cellular resistance to gemcitabine between acquired gemcitabine resistant MiaPaCa2-RG cells and PSN1-RG cells. Next, we examined the correlation of RRM1 mRNA expression levels with clinical course of 18 patients with recurrent pancreatic cancer. Seven patients developed liver metastasis, 4 developed local recurrence, 3 developed lymph node metastasis, 2 developed lung metastasis and 2 developed multi site recurrence (1 patient had local recurrence and liver metastasis and the other had liver, lung, bone and lymph node metastasis). The response to gemcitabine were CR (n = 0), PR (n = 2), SD (n = 6) and PD (n = 10). We classified 8 patients as responders (PR and SD) and 10 patients as nonresponders (PD). On the other hand, the median RRM1 mRNA expression relative to the housekeeping gene PBGD was 1.3×10^{-2} (minimum expression, 0.0×10^{-2}; maximum expression, 132.0×10^{-2}) in 18 pancreatic tissue samples (Fig. 7). According to a cut-off value of 1.3×10^{-2}, 9 patients (50%) were classified into the low RRM1 expression group, and 9 patients (50%) into the high RRM1 expression group. There was a significant association between gemcitabine response and RRM1 expression (p = 0.018) (Table III). Furthermore, patients with

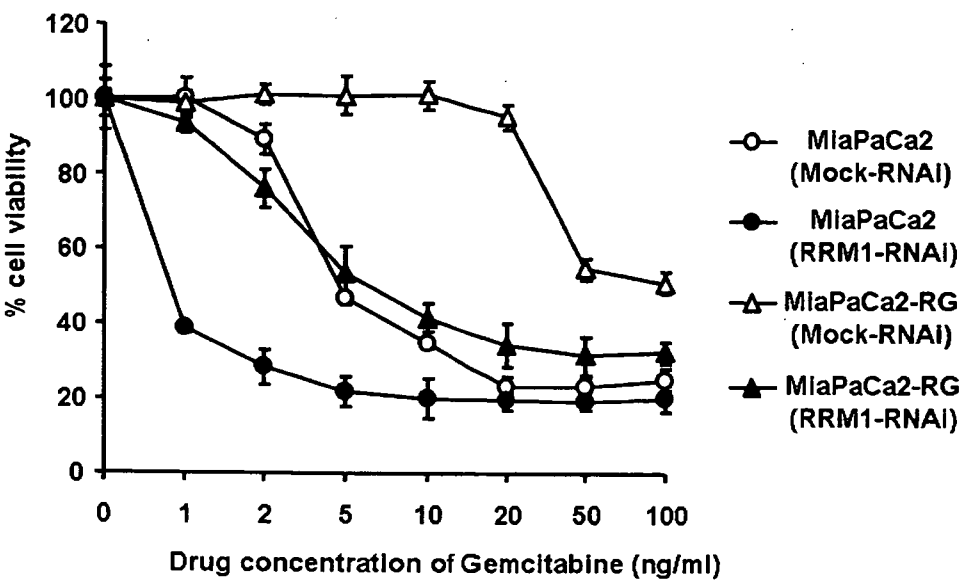


FIGURE 5 – Dose–response curves for gemcitabine in MiaPaCa2 (circle) and MiaPaCa2-RG (triangle) after RNAi transfection. Open circles and triangles indicate mock RNAi transfectant. Closed circles and triangles indicate RRM1-specific RNAi transfectant. Points, mean; bars, SD (n = 3).

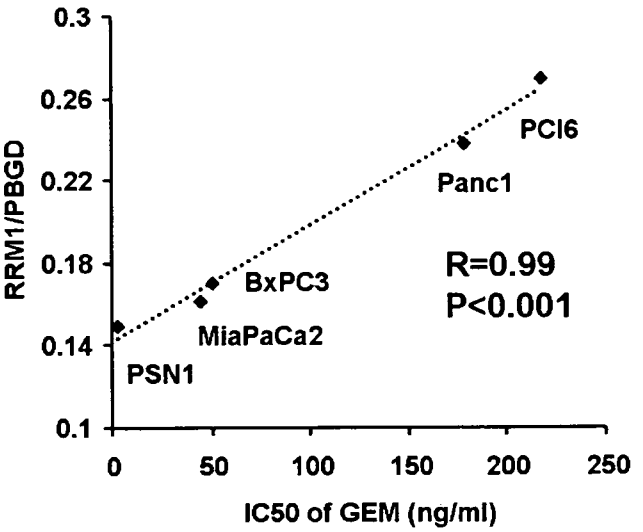


FIGURE 6 – Correlation of gemcitabine sensitivity with RRM1 expression in 5 human pancreatic cancer cell lines ($n = 3$). $R = 0.99$, $p < 0.001$.

high RRM1 levels had poor survival times after gemcitabine treatment than those with low RRM1 levels (Fig. 8; $p = 0.016$). Median survival times after gemcitabine treatment was 6.0 months for patients with high RRM1 levels and 14.6 months for patients with low levels.

Discussion

The present study have demonstrated that RRM1, which is a subunit of ribonucleotide reductase (one of the key enzymes in gemcitabine metabolism), should be clearly involved in gemcitabine resistance in human pancreatic cancer. First, oligonucleotide microarray analysis covering 30,000 human oligonucleotides between human pancreatic cancer cells resistant to gemcitabine and parental cells demonstrated that the most upregulated gene in the gemcitabine-resistant variant MiaPaCa2-RG cells was the RRM1 gene. RRM1 expression in the resistant cells was 4.5-fold higher than parental cells. This up-regulation was validated by quantitative RT-PCR and Western blotting. Furthermore, there was no difference between the expression levels of the other subunits of ribonucleotide reductase or the other molecules in gemcitabine metabolism including dCK, CTP synthetase, dCMP deaminase and nucleotide transporters. Second, by RRM1-specific RNAi transfection, RRM1 expression in both mRNA and protein levels were significantly decreased and the gemcitabine chemoresistance of MiaPaCa2-RG was significantly reduced to same level as that of MiaPaCa2. Third, the most important point was confirmation by the clinical analysis. Increased RRM1 expression was significantly associated with antitumor effects and with poor survival after treatment with gemcitabine in pancreatic cancer patients ($p = 0.018$ and 0.016, respectively). Therefore, RRM1 could be the targeted molecule to regulate gemcitabine resistance. Furthermore, its expression levels could be a useful indicator of gemcitabine resistance.

Ribonucleotide reductase (RR) acts as the rate-limiting enzyme in *de novo* DNA synthesis, because it is the only known enzyme that converts ribonucleotides to deoxyribonucleotides, which step is mandatory for DNA polymerization and repair.[31,32] In the cell, a deoxycytidine analogue, gemcitabine, is phosphorylated to monophosphate, diphosphate, and triphosphate before incorporation into DNA, which is required for its growth inhibiting activity. The diphosphorylated form of gemcitabine acts as a RR inhibitor, and some of gemcitabine cytotoxic activity is due to this inhibition.[33] Ribonucleotide reductase increases the deoxynucleoside

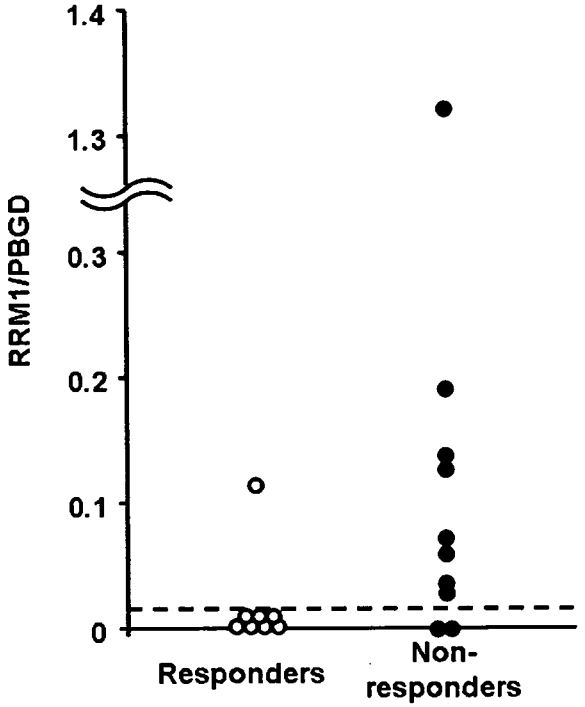


FIGURE 7 – RRM1 expression levels classified by gemcitabine response in the human pancreatic cancer tissues. Dotted bar: RRM1 cut-off value of 1.3×10^{-2}.

TABLE III – ASSOCIATION BETWEEN GEMCITABINE RESPONSE AND RRM1 mRNA EXPRESSION LEVELS

	RRM1 level[1]		
	High	Low	Total
Responder (PR, SD)	1	7	8
Nonresponder (PD)	8	2	10
Total	*9*	*9*	*18*

Fisher's exact test, $p = 0.018$.
[1]Cut-off value is median RRM1 expression relative to PBGD (1.3×10^{-2}).

triphosphate (dNTP) pool in the cells, which could lead to decreased incorporation of dNTP analogues such as triphosphorylated gemcitabine into DNA and might reduce the antitumor effect of gemcitabine.[22] In fact, MiaPaCa2-RG, higher expresser of RRM1 mRNA, showed lower gemcitabine uptake than lower RRM1 expresser MiaPaCa2.

Recent results have shown that there are 3 human ribonucleotide reductase subunits: RRM1, RRM2 and p53R2. RRM1 is a large peptide chain (α), and RRM2 and p53R2 are small protein subunits of RR (β). The catalytically active form of eukaryotic ribonucleotide reductase is proposed to be a $\alpha_2\beta_2$ heterotetrameter made up of 2 large subunits and 2 small subunits.[34,35] Although ribonucleotide reductase enzymatic activity is modulated by levels of RRM2[36] and p53R2,[37] RRM1 could play a key role among the 3 subunits in the course of gemcitabine treatment. RRM1 controls substrate specificity and global on/off enzyme activity.[36,37] As suggested by Davidson *et al.*, RRM1 could act as a "molecular sink" for gemcitabine, in which RRM1 binds irreversibly to the drug and inactivates it, while increased RRM1 expression did not alter ribonucleotide reductase activity in the gemcitabine resistant variant human lung cancer cells.[12] RRM1 was upregulated in the 2 selected gemcitabine resistant human lung cancer cell lines, where RRM1 expression levels were correlated with gemcitabine concentration for cell selection.[12] A recent microarray analysis