In the group with surgical resection, SCN was correctly diagnosed in only 1 of 7 patients who underwent preoperative biopsy (14.3%). Therefore, it may be difficult to achieve an accurate pathological diagnosis of SCN using a biopsy specimen obtained by fine needle aspiration. Open laparotomy biopsy or True-Cut biopsy may be superior to fine needle biopsy in the diagnosis of SCN.

Operative Procedures for SCN

Various surgical procedures were performed according to the location of SCN. This is because of the policies of the respective institutions; for example, whether an organ-preserving procedure is applied. Because lymph node metastasis of SCN is quite rare, an organ-preserving surgical procedure is recommended for ordinary SCN.[8] The use of laparoscopic operations for SCN may increase in number.

Surgical Indications for SCN According to Malignancy

The mean tumor size in the present series was 4.1 (2.8) cm. Kimura and Makuuchi,[16] Kimura,[20] and Kimura et al[29] reported that SCN larger than 4 cm should be considered for operation. Pathological findings that suggested malignant SCN in our patients included nuclear atypia, papillary proliferation,[20] lymph vessel invasion, venous invasion, and peripancreatic fat tissue infiltration. Because there was no mortality associated with SCN, it was unclear whether these pathological findings were correlated with the prognosis. Some authors have reported lymph node metastases.[19,33] Abe et al[33] reported a case of SCN infiltration in peripancreatic fat tissue. Kimura and Makuuchi,[16] Kimura,[20] and Kimura et al[29] reported "interstitial infiltration" in 2 SCN lesions larger than 5 cm. Kimura and Makuuchi,[16] Kimura,[20] and Kamei et al[11] reported perineural invasion. There have been many reported cases that have suggested malignancy.[9–20] The Johns Hopkins group reported 1 liver metastasis and 3 locally advanced SCN.[8] In 1 of these 4 patients, recurrence of SCN was observed in the liver and retroperitoneum 14 years after resection. The estimated rate of malignancy for SCN in their series is approximately 1%.

Whether SCN can unconditionally be observed or not is a practical problem. The number of followed up patients is increasing, probably because of increased accuracy of preoperative diagnosis of SCN. We experienced 2 patients with SCN with liver metastasis. There is no consensus for surgical indication according to the size of the tumor. However, rupture of gastric varices,[34,35] obstructive jaundice,[35,36] and gastrointestinal bleeding due to invasion[37] have been noted. Therefore, surgical resection should be performed for symptomatic SCN. Hashimoto et al[32] reported that tumors of the pancreas that cannot be confirmed to be SCN should be resected because of the possibility of other neoplasms with malignant potential. In this study, the 2 patients with liver metastasis had microcystic-type and solid-type SCNs. Moreover, both cases tended to be large, with a size of 15 cm; liver metastasis may occur in large SCN cases.

With regard to clinicopathological features, the observation group tended to be older than the group with surgical resection. As expected, the proportion of symptomatic patients was significantly greater in the group with surgical resection ($P < 0.05$). The diameter of SCN in the observation group tended to be smaller (mean, 3.7 cm) than that in the group with surgical resection (mean, 4.4 cm). Kimura and Makuuchi[16] reported that surgery was indicated for SCN larger than 4 cm. A similar result was observed in this study. The honeycomb appearance, typical for the microcystic SCN, was frequently noted in the observed group just as expected. In contrast, observation might be recommended for SCN in elderly which size is small and shows the typical honeycomb appearance.

This study had the following characteristics or limitations: (1) Patients who were operated on for suspected SCN, but the postoperative diagnosis was not SCN, were not included in this study. (2) The observation group might have included some patients with non-SCN. (3) Patients operated on under the presumptive diagnosis of non-SCN after differentiation of SCN and whose tumor was not finally SCN were not included.

CONCLUSIONS

In patients with suspected SCN, surgical resection should be considered (1) when differentiation from other neoplasms such as IPMN, MCN, endocrine tumor, and pancreatic cancer is difficult; (2) when the patient has symptoms or mass effects to the main pancreatic duct; and (3) when the tumor size is large or increasing.

In contrast, a simple observation of the clinical course might be recommended for probable SCN in elderly patients or which is small and showing the typical honeycomb appearance but no communication or compression to the main pancreatic duct.

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Combination therapy of gemcitabine or oral S-1 with the anti-VEGF monoclonal antibody bevacizumab for pancreatic neuroendocrine carcinoma

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Abstract. We previously reported that the administration of bevacizumab for pancreatic neuroendocrine tumors inhibited angiogenesis in the host, resulting in tumor growth inhibition. In light of these results, we compared the effect of bevacizumab/gemcitabine/S-1 combination therapy vs. bevacizumab monotherapy. The QGP-1 pancreatic neuroendocrine carcinoma cell line and the BxPC-3 ductal cell carcinoma cell line were transplanted into the subcutaneous tissue of mice, and the mice were treated for 3 weeks with bevacizumab [50 mg/kg intraperitoneally (i.p.) twice weekly], gemcitabine (240 mg/kg i.p. once weekly) and S-1 (10 mg/kg orally five times weekly). The antitumor effect and side effects were evaluated by measuring the tumor volume and weight and by changes in body weight, respectively. The tumor volume became smaller (from the maximum volume) in the group treated with bevacizumab, gemcitabine and S-1 (BGS) and the group treated with bevacizumab and gemcitabine (BG). A significant difference was noted in the tumor weight between the BG group and the group treated with bevacizumab alone. A relatively significant decrease in the body weight was observed in the BGS and BG groups. We conclude that gemcitabine is appropriate as a drug used in combination with bevacizumab for pancreatic neuroendocrine tumors.

Introduction

Both functional and non-functional pancreatic neuroendocrine tumors (PNETs), including pancreatic neuroendocrine carcinomas (PNECs), are hypervascular tumors and they are known to express angiogenic molecules (1,2). For these reasons, anti-angiogenic therapy is expected to be effective against PNEC (3). Bevacizumab (Avastin®; Genentech Inc., San Francisco, CA, USA) is a recombinant human IgG1 monoclonal antibody against vascular endothelial growth factor (VEGF) (4). We previously reported that bevacizumab inhibited the induction of host angiogenesis, resulting in significant tumor growth inhibition, but not in tumor cell proliferation using QGP-1 which is a PNEC cell line, and expected a further potent cytotoxic effect by various combinations with anticancer drugs (5). On the basis of the suggestion above, we compared an additional effect between the combination of gemcitabine hydrochloride (Gemzar®, Eli Lilly and Company, Indianapolis, IN, USA) (6) or oral S-1 (TS-1®, Taiho Pharmaceutical Co. Ltd., Tokyo, Japan) (7) with bevacizumab and bevacizumab alone.

Materials and methods

The QGP-1 PNEC cell line (8) was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan), and the BxPC-3 human pancreatic ductal carcinoma (DCC) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C in RPMI-1640 (Gibco, Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO_2.

Athymic female Balb/c-nu/nu nude mice (4-6 weeks old) with a body weight (BW) of 20-22 g, obtained from Clea Japan Inc. (Tokyo, Japan), were kept at the Animal Care and Use Facilities at Tokyo Medical University under specific pathogen-free condition. The cell suspension of each cell line with an adjusted cell suspension of $2x10^7$ cells/ml in RPMI-1640 (Gibco) was mixed with Matrigel matrix (BD Biosciences, San Jose, CA, USA) on ice at a 1:4 ratio. The mixture was implanted subcutaneously in the back of mice. At predetermined time points during a 1-week period after the cancer transplantation, 25 mice were randomly divided into five groups and treated with bevacizumab and gemcitabine or S-1 for 3 weeks. Bevacizumab (4 mg/kg) or human IgG (Sigma) was administered intraperitoneally (i.p.) twice a week (9). Gemcitabine (240 mg/kg) was administered i.p. once a week (10). Hydroxypromethyl cellulose [0.2 ml of

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0.5% (w/v); Shin-Etsu Chemical Co., Ltd., Tokyo, Japan], including dissolved powder-form S-1 (10 mg/kg) were orally administered five times a week (11,12). The treatment groups were as follows: BGS group, mice received bevacizumab, gemcitabine and S-1; BG group, mice received bevacizumab and gemcitabine; BS group, mice received bevacizumab and S-1; B group, mice received bevacizumab alone; and IgG group, mice received human IgG as non-treatment. Tumor volume was calculated by the multiplication of π x longitudinal axis x minor axis x minor axis; measurement was carried out using digital calipers, once a week. The weight of the mice was measured once a week. On the last day of the third week after start of the therapies (on 28 day after cancer cell transplantation), each tumor was removed and weighed. All experiments were approved by the Animal Care and Ethics Committee of Tokyo Medical University.

Statistical analysis. Statistical analyses were performed using Stat View (Abacus Concepts Inc., Berkely, CA, USA). The volume of the tumor was compared using the Mann-Whitney U test. A two-side p-value of <0.05 was considered to denote statistical significance.

Results

BxPC-3 cell tumors grew to approximately double that of the QGP-1 cell tumors. The mean tumor volume (mm^3) 1 week after QGP-1 transplantation, and 1, 2 and 3 weeks after each treatment was as follows: for the BGS group: 300.6, 389.8, 567.6 and 442.2, respectively; for the BG group: 486.8, 531.8, 546.3 and 358.2, respectively; for the BS group: 381.4, 436.1, 638.9 and 725.6, respectively; for the B group: 462.0, 549.7, 970.4 and 949.9, respectively; and for the IgG group: 414.2, 607.0, 935.4 and 1,504.2, respectively (Fig. 1A). The BGS and the BG groups receiving gemcitabine showed marked tumor growth inhibition from the 2nd week or later. By contrast, the BS and the B groups not receiving gemcitabine showed tumor growth inhibition in comparison to the IgG group; however, the tumor increased after the 2nd week or later. The tumor volume of all treatment groups apart from group B at the 3rd week was significantly smaller than that of the IgG group ($p<0.05$). There was no significant difference among the treatment groups. The mean tumor weight in the BGS, BG, BS, B and IgG groups at the time of tumor dissection was 382.9, 515.5, 114.7, 532.8 and 1,653.6 mg, respectively. There was a significant difference between all treatment groups and the IgG group ($p<0.05$), and between the BS and the B group ($p=0.03$) (Fig. 1B).

The mean tumor volume (mm^3) of the BxPC-3 cell tumors was as follows: for the BGS group: 546.1, 527.5, 473.0 and 496.9, respectively; for the BG group: 567.4, 639.7, 528.8 and 475.8, respectively; for the BS group: 437.7, 665.5, 447.1 and 347.7, respectively; for the B group: 526.6, 493.6, 341.8 and 523.6, respectively; and for the IgG group: 743.7, 1,243.1, 2,350.8 and 2,991.2, respectively (Fig. 2A). The BGS, BG and BS groups showed slight tumor inhibition from the 2nd week or later; however, only the B group exhibited tumor growth. The tumor volume of all treatment groups at the 3rd week was significantly smaller than that of the IgG group ($p<0.05$), but not among the treatment groups. The mean tumor weight in the BGS, BG, BS, B and IgG groups was 339.2, 325.7, 217.2, 322.8 and 1,782.7 mg, respectively. There was a significant difference between all treatment groups and the IgG group ($p<0.05$), but not among the treatment groups (Fig. 2B).

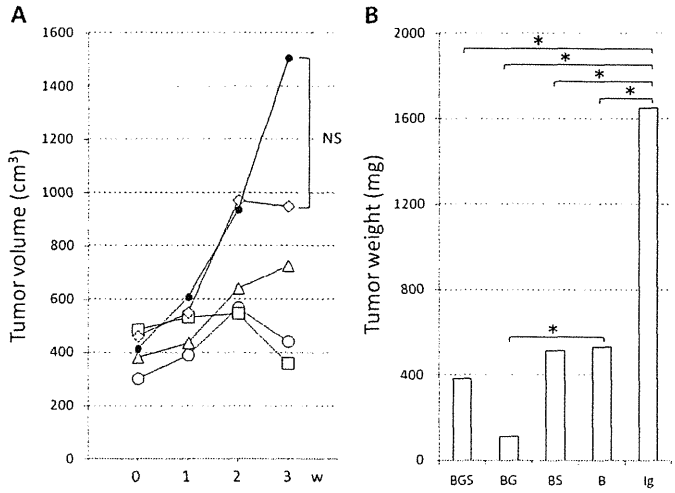

Figure 1. (A) Change in tumor volume with serial time course of QGP-1. x-axis: the time period (weeks). 0 (w) is the start day of therapy (1 week after transplantation). y-axis: tumor volume (cm^3). Tumor volume of mice administered: •, human IgG (Ig); ◇, bevacizumab alone (B); △, bevacizumab and S-1 (BS); ○, bevacizumab and gemcitabine (BG); and □, bevacizumab, gemcitabine and S-1 (BGS). Significant difference between the IgG group in all treatment groups except the B group was noted. (B) Final tumor weight after treatment. x-axis: treatment groups. y-axis: tumor weight (mg). *Significant difference between the untreated group and all treatment groups, and between B and BG group is shown.

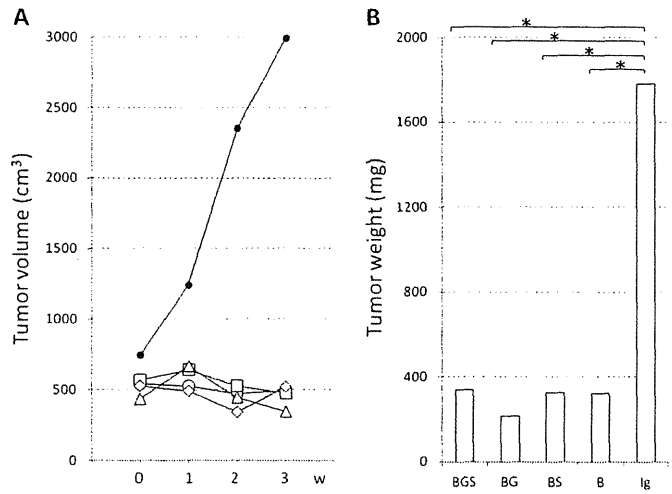

Figure 2. (A) Change in tumor volume with serial time course of BxPC-3. x-axis: the time period (weeks). 0 (w) is the start day of therapy (1 week after transplantation). y-axis: tumor volume (cm^3). •, Ig group; ◇, B group; △, BS group; ○, BG group; □, BGS group. Significant difference was noted between the IgG group and all treatment groups. (B) Final tumor weight after treatment. x-axis: treatment groups. y-axis: tumor weight (mg). *Significant difference was noted between the IgG group and all treatment groups.

Macroscopic findings of the QGP-1 cell tumors showed comparatively solid and little central necrosis and no marked differences among the treatment groups (Fig. 3A-C). By contrast, the macroscopic findings of the BxPC-3 cell tumors indicated intratumoral bleeding and necrosis in all groups (Fig. 3D-F). Numerous subcutaneous blood vessels were

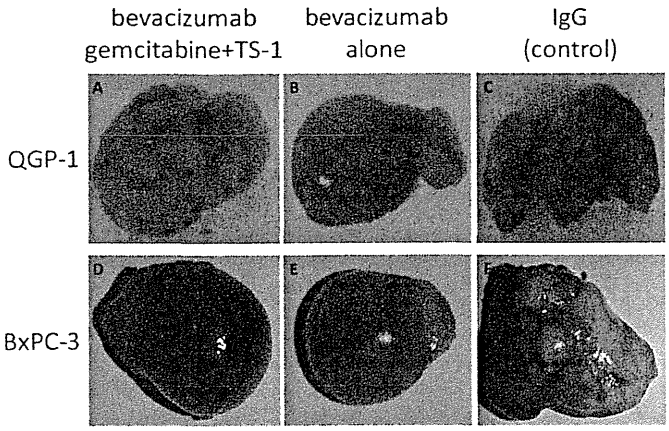

Figure 3. Macroscopic features of subcutaneous (A-C) QGP-1 cell and (D-F) BxPC-3 cell tumors. (A and D) Tumors treated with bevacizumab, gemcitabine and TS-1. (B and E) Tumors treated with bevacizumab alone. (C and F) Tumors treated with IgG as a control. As for QGP-1 cell tumors, the surface was yellowish white, and the inside of the tumor was solid. By contrast, for BxPC-3 cell tumors, the surface exhibited a reddish tinge, and the treated tumors (D and E) showed internal hemorrhage and necrosis.

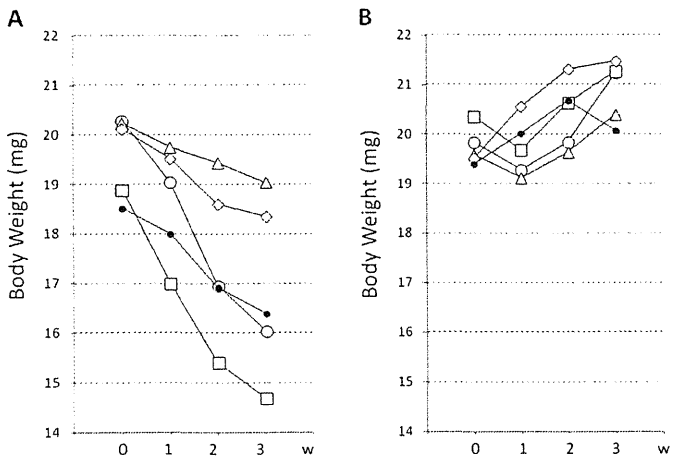

Figure 4. Body weight of mice bearing (A) QGP-1 and (B) BxPC-3 cell tumors. x-axis: time course (weeks). y-axis: body weight (mg). As for the weight change in the QGP-1 cell-transplanted mice, all groups, especially the BGS and BG groups, showed weight loss. By contrast, the body weight of mice bearing the BxPC-3 cell tumors was increased. •, Ig group; ◇, B group; △, BS group; ○, BG group; □, BGS group.

overlying the tumors in the IgG group bearing the QGP-1 and BxPC-3 cell tumors, while few blood vessels were observed in the bevacizumab-treated group.

As for the weight change in the QGP-1 cell-transplanted mice, all groups showed weight loss. In the BGS and BG groups, the drug caused weight loss which was in particular stronger than that in the IgG group. By contrast, weight loss was not evident, but weight instead rather increased in the BxPC-3 cell-transplanted mice. We determined that the above results reflected solely the characteristics of the cell lines.

Discussion

Inhibition of angiogenesis has become a target of cancer therapy, and the anti-VEGF antibody/bevacizumab is representative. Bevacizumab specifically binds to VEGF in the bloodstream and inhibits the binding of VEGF to VEGF receptors in vascular endothelial cells, thereby inhibiting angiogenesis. The interstitial pressure around a tumor is usually increased, inhibiting the delivery of anticancer drugs to tumor tissue. Bevacizumab normalizes tumor blood vessels, reduces the interstitial pressure and thereby improves the delivery of anticancer drugs to tumor tissue (4). PNECs are also hypervascular tumors and are known to express angiogenic molecules (1-3). For these reasons, anti-angiogenic therapy is expected to be effective against PNEC. In a randomized phase II trial of bevacizumab vs. interferon-α for the treatment of patients (n=44) with unresectable carcinoid tumors treated with octreotide, a somatostatin analogue, the added effect of combining bevacizumab with the somatostatin analogue, was reported (13). The therapeutic response rates were 18 vs. 0%, and the 8-week progression-free survival rates were 95 vs. 68%. We previously reported that bevacizumab inhibited the induction of host angiogenesis, resulting in significant tumor growth inhibition (5).

In the selection of therapeutic agents, we focused on the site of origin and growth of PNEC. PNECs are considered to arise from Langerhans cells, endocrine acinar cells and multipotent stem cells in the pancreatic ducts. By contrast, it has been reported that pancreatic ductal cell carcinoma may arise from pancreatic endocrine cells (14). In addition, Langerhans cells or pancreatic endocrine cells are reportedly involved in the growth of pancreatic ductal cell carcinoma (14). In light of these observations, we selected gemcitabine and S-1, which are therapeutic agents for pancreatic ductal carcinoma, as candidate therapeutic agents for PNEC, and confirmed a more beneficial effect of gemcitabine/bevacizumab combination therapy over bevacizumab monotherapy. Concerning the combination treatment of gemcitabine and bevacizumab, a randomized controlled trial of gemcitabine + placebo vs. gemcitabine + bevacizumab for the treatment of advanced unresectable pancreatic cancer was conducted. However, no significant differences were observed between the gemcitabine + placebo and gemcitabine + bevacizumab groups in the therapeutic response rates, median progression-free survival times and median survival times. Thus, gemcitabine + bevacizumab therapy did not prolong the survival time compared to gemcitabine therapy (15). On the contrary, a case report of the utility of the combination therapy including bevacizumab and gemcitabine for the progression of pancreatic cancer was reported (16). Another candidate therapeutic agent, S-1, was first developed in Japan (7,17). Currently, gemcitabine and S-1 are the only drugs that contribute to improving the prognosis of pancreatic cancer. Either gemcitabine or S-1 is commonly used as a first-line treatment, but they are sometimes used in combination with each other (18). Combination therapy with S-1, irinotecan and bevacizumab has been reported to be useful in the treatment of colorectal cancer with metastasis (19). In this study, we expected to obtain better results using a combination therapy with bevacizumab, gemcitabine and S-1, and confirmed a more beneficial effect of bevacizumab/gemcitabine combination therapy over bevacizumab monotherapy. However, the triple therapy was not superior to bevacizumab/gemcitabine combination therapy in the QGP-1 cell-transplanted mice.

The effect of the mammalian target of rapamycin (mTOR) inhibitor everolimus (Afinitor®) in patients with

advanced pancreatic neuroendocrine tumors has recently been reported (20). In this clinical trial, treatment with the mTOR inhibitor extended the median survival time from 4.6 (in a placebo group) to 11 months (in the treated group). It was also found that the mTOR inhibitor exerted an angiogenesis-inhibitory effect through VEGF (21). Future research will be conducted to investigate how to combine drugs for the treatment of pancreatic neuroendocrine tumors.

In conclusion, we compared the effect of bevacizumab/gemcitabine/S-1 combination therapy vs. bevacizumab monotherapy on pancreatic neuroendocrine tumor cell lines. Bevacizumab/gemcitabine combination therapy showed a strong antitumor effect (a decrease from the maximum tumor volume) from 2 weeks after treatment initiation. By contrast, bevacizumab/S-1 combination therapy resulted in a slowdown of tumor growth, but not in a decrease from the maximum tumor volume. Thus, we conclude that gemcitabine is appropriate for use in combination with bevacizumab for pancreatic neuroendocrine tumors.

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Original Paper

Prediction of a Side Effect and Efficacy of Adjuvant Chemotherapy with Gemcitabine for Post Operative Patient of Pancreatic Cancer by a Genetic Polymorphism Analysis

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ABSTRACT

Background/Aims: Single nucleotide polymorphism (SNP) of the genes for ATP-binding cassette transporters is related to the side effects of anticancer drugs and that of drug metabolism-related enzyme genes is involved in the activation of gemcitabine (GEM). **Methodology:** Forty eight patients treated with adjuvant GEM chemotherapy after pancreatic cancer resection was examined for the SNP of multidrug-resistance 1 (MDR1) 2677, MDR1 3435, breast cancer resistance protein (BCRP) 421, ribonucleotide reductase M1 (RRM1)(-)524, RRM1(-)37 and deoxycytidine deaminase (CDA) 208. We divided the patients according to normal group: patients homozygous for a wild-type allele or heterozygous for a mutant allele and mutant group: those homozygous for a mutant allele. Both groups were compared regarding the outcome and the occurrence and severity of side effects. **Results:** MDR1 2677, MDR1 3435, BCRP421, RRM1(-) 524, RRM1(-) 37 and CDA mutant groups comprised 37.5, 31.3, 0, 12.5, 4.2 and 4.2%, respectively. The occurrence of ≥G3 side effects was the most frequent in the MDR1 2677 mutant group at 39%. The disease-free survival and overall survival tended to be longer in the MDR1 2677 mutant group. **Conclusions:** A correlation between the SNP of MDR1 2677 and drug response in patients receiving GEM chemotherapy.

Key Words: Gemcitabine; Pancreatic cancer; Single nucleotide polymorphism; Multidrug-resistance 1.

Abbreviations: Multidrug-Resistance 1 (MDR1); Breast Cancer Resistance Protein (BCRP); ATP-Binding Cassette Sub-Family G Member 2 (ABCG2); Ribonucleotide Reductase M1 Gene (RRM1); Deoxycytidine Deaminase Gene (CDA).

INTRODUCTION

Pancreatic cancer is difficult to diagnose early and almost all patients are diagnosed at an advanced stage. Surgical resection is the only hope for cure; however, even R0 resection is associated with a high recurrence rate. Therefore, improvement based on the long-term results of surgery alone is limited, necessitating the additional use of adjuvant chemotherapy (1). Since the dose reduction or discontinuation of anticancer drugs significantly affects the prognosis, it is necessary to adequately control the side effects. As side effects may markedly reduce the quality of life, particularly in patients after highly invasive surgery, such as pancreatic cancer surgery, they should be more carefully monitored. It is known that cell membrane-localized ATP-binding cassette (ABC) transporters are involved in the development of side effects and cancer drug resistance (2). In particular, it has been shown that single nucleotide polymorphism (SNP) is associated with the reduced activity of transporters, leading to excessive side effects. Among the SNPs of the ABC transporter genes, those of the multidrug-resistance 1 (MDR1) (3-7) and breast cancer resistance protein (BCRP), also known as ATP-binding cassette sub-family G member 2 (ABCG2) (8,9) genes have been most widely studied. The deoxycytidine analogue gemcitabine (GEM), the standard therapeutic agent for pancreatic cancer, is a prodrug which is phosphorylated *in vivo* by the drug metabolism-related enzyme deoxycytidine kinase (dCyd kinase) to its active form, 2',2'-difluorodeoxycytidine-5'-triphosphate (dFdCTP) (10,11). It has been reported that dCyd kinase activity varies among individuals and that the SNP of drug metabolism-related genes is related to the incidence of side effects and drug efficacy and resistance. In particular, the SNP of the ribonucleotide reductase (RR) M1 gene (RRM1) (12-17) and deoxycytidine deaminase gene (CDA) (18-20) has been most widely studied.

In recent years, the practical application of individualized drug therapy, so-called tailor-made medicine has advanced. In particular, the inexpensive analysis of host SNP can be a major source of information on drug responsiveness in cancer patients, whose numbers have been rapidly increasing. The prediction of drug side effects and making a prognosis, especially in the field of adjuvant chemotherapy, may offer the possibility of avoiding the unnecessary administration of anticancer drugs. In this study, we investigated the presence or absence of SNP of the drug metabolism-related genes MDR1 2677, MDR1 3435, BCRP421, RRM1(-)524, RRM1(-)37 and CDA208 and its

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correlation with adverse events and the prognosis in patients who had received adjuvant chemotherapy after pancreatic cancer surgery.

METHODOLOGY

Patients

Thirty nine patients who received adjuvant GEM treatment after pancreatectomy for pancreatic cancer in Tokyo Medical University Hospital between June 2004 and March 2008 and 9 in Hachioji Medical Center of Tokyo Medical University between April 2008 and September 2009 were included in this study. Patient characteristics and pathological stage of their tumor (21) have been described in detail in **Table 1**. This study was performed with the approval of the medical ethics committee of our university and after obtaining informed consent from all subjects.

Gemcitabine medication

GEM were given at day 1 and day 15 of 4 weeks for patients under R=0 or R=1 resection. Patients with ≥ grade 3 (≥ G3) side effects according to the Common Terminology Criteria for Adverse Event v3.0 (CTCAE v3) were given a granulocyte-colony stimulating factor or an anti-nausea drug on all such occasions. Patients under R=0 or R=1 resection received GEM medication of full dose until recurrence.

Evaluation of single nucleotide polymorphism

Genomic DNA was isolated from peripheral lymphocytes from venous blood (2mL) of patients with using QIAamp DNA Blood Kit (QIAGEN Inc., Valencia, CA, USA). Target site of DNA was amplified by polymerase chain reaction (PCR) which was performed a total volume of 50μL in the presence of 100μg of cDNA, SuperMix (Invitrogen Corp., Carlsbad, CA, USA) and 0.25μM each primers as shown at **Table 2**. After an initial denaturation, 35 cycles of 10 sec at 98°C, 30 sec at 55°C and 1 min at 72°C, 5 min at 68°C as well as a final extension period were carried out. The mutations in genes MDR1 2677, MDR1 3435, BCRP421, RRM1(-)524, RRM1(-)37 and CDA208 were confirmed by a DNA sequences directly on a 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA) using a Big-Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems). The sequencing primers were those used in the PCR amplifications. The sequence of all PCR products about MDR1 2677, 352 base pairs (bp) were analyzed by DNA sequences directly to confirm G to C, T or A mutation. Some PCR products except MDR1 2677 were examined in each sequence and the following restriction fragment length polymorphism (RFLP) was performed. We confirmed the agreement of both results afterwards. Each PCR product was digested by an appropriate restriction enzyme (New England Biolabs Inc., Ipswich, MA, USA), MboI for MDR1 3435, MseI for BCRP421, ApoI for RRM1(-)524, BbSI for RRM1(-)37 and RsrII for CDA208, under its standard condition to screen SNP of each target gene. SNP was confirmed by RFLP under electrophoresis on 5% agarose gel to check size of fragments at 150V of constant power for 1 to 4 hours depending on fragment size. We confirmed these results by direct DNA sequencing.

TABLE 1. Patient characteristics.

Patient characteristics	Number of patients
Age (years), median (range)	68 (48-85)
Gender, Male:female	30, 18
Clinical staging	
Stage I	1
Stage II	2
Stage III	28
Stage IVa	12
Stage IVb	5
Operation	
Pancreaticoduodenectomy: PD	
Pylorus preserving pancreaticoduodenectomy: PPPD	37
Distal pancreatectomy: DP	11

Statistical analysis

Statistical analyses were performed using StatView (Abacus Concepts Inc., Berkeley, CA, USA). The overall survival (OS) and disease-free survival (DFS) probabilities were estimated using the Kaplan-Meier method with log-rank tests from the initial date of surgical resection. A two-sided p value of <0.05 was considered to represent a statistically significant difference.

RESULTS

All 48 patients had the tumor surgically resected and pathologenic evaluation of the surgical specimens showed negative or a microscopically positive margin (R0, or R1 resection). The average and standard deviation (SD) until an initial GEM administration day was 57.3 (±39.3) postoperative days (range 15-210 days). The average number of the GEM administration was 21.0 (±17.3) times. The amount of the GEM administration was from 2,600 to 130,200mg and the average was 26,387.5mg. The mean DFS was 1.48 (±1.38) years (range 3.3 months-5 years 7 months) and the mean OS was 1.72 (±1.39) years (range 5.5 months-5 years 7 months) (**Table 3**).

The frequencies of SNP of each gene are shown in **Table 4**. Homozygous wild-type and heterozygous mutant individuals were classified as a normal group and homozygous mutant individuals as a mutant group. Patients 18

TABLE 2. PCR primers of each gene.

Target gene	Primer
MDR1 2677	F 5'-agaagcatgagttgtgaaga-3'
	R 5'-gcatagtaagcagtagggag-3'
MDR1 3435	F 5'-tgatggcaaagaaataaagcg-3'
	R 5'-tgactcgatgaaggcatgtatgt-3'
BCRP421	1st PCR
	F 5'-caggttacgtggtaca-3'
	R 5'-agtggcagactccaag-3'
	2nd PCR
	F 5'-gccttaaggatgatgt-3'
	R 5'-acaactatgacgaatc-3'
RRM1(-)37	F 5'-gtagtcttctgggtcttgcc-3'
	R 5'aaaggggcgcgacggggttc-3'
RRM1(-)524	F 5'-gtcaccaagtccatcctac-3'
	R 5'-cgagaaggaaggttaaggg-3'
CDA208	F 5'-aatctaccagtgcccca-3'
	R 5'-gagtgctgaggataaggag-3'

F: Forward primer, R: Reverse primer.

and 15 who were a mutant homozygote for MDR1 2677 and MDR1 3435 classified as MDR1 2677 and MDR1 3435 mutants respectively. No patient was included in the mutation group of BCRP421. Patients 6 and 2 who were a mutant homozygote for RRM1(-)524 and RRM1(-)37 classified as RRM1(-)524 and RRM1(-)37 mutants, respectively. Two patients who were a mutant homozygote for RRM1(-)524) were included in mutant homozygotes for RRM1(-)37. All 22 patients with wild-type RRM1(-)524 had wild-type RRM1(-)37. Thus, the presence or absence of these SNPs was in agreement with a high probability. CDA208 mutants accounted for 4.3% (2/48).

The frequencies of SNPs of each gene and G3 or higher side effects are shown in **Table 5**. Side effects were observed in 23% (11/48) of all patients. The rate of G3 or higher side effects in MDR1 2677 mutant subjects who were expected to develop severe side effects was 39%, higher than the 13% in the normal group. In contrast, those of MDR1 3435 were 13%, lower than the 27% in the normal group. G3 or higher side effects were not shown in all mutant groups of BCRP421, RRM1(-)524, RRM1(-)37 and CDA208.

The DFS and OS tended to be longer in the MDR1 2677 mutant group (the mean total amount of GEM administered, 35,488.89mg; normal, 31,046.82mg) (p=0.175 and p=0.298, respectively; no significant difference) (**Figure 1**), but not in the MDR1 3435 mutant group (p=0.875 and p=0.302, respectively; no significant difference) (**Figure 2**).

TABLE 3. GEM administration.

	Average ±SD	Range
Postoperative days of GEM start	57.3±39.3	15-210
Number of GEM doses	21.0±17.3	3-93
Amount of GEM dosage (mg)	26387.5±23813.9	2,600-130,200
Disease free survival (y)	1.48±1.38	3.3 m-5y7m
Overall survival (y)	1.72±1.39	5.5 m-5y7m

SD: Standard Deviation.

TABLE 4. Single nucleotide polymorphism (SNPs) genotype distribution.

	Normal group		Mutant group
	Wild type	Heterogeneous-mutation	Homogeneous-mutation
MDR1 2677	8 (G/G)	22 (G/T,A)	18 (T/T, T/A)
MDR1 3535	17 (C/C)	16 (C/T)	15 (C/T)
BCRP 421	47 (C/C)	1 (C/A)	0 (A/A)
RRMI(-)524	22 (T/T)	20 (T/C)	6 (C/C)
RRMI(-)37	25 (C/C)	21 (C/A)	2 (A/A)
CDA 208	42 (G/G)	4 (G/A)	2 (A/A)

TABLE 5. Adverse events and single nucleotide polymorphisms (SNPs).

	Normal group		Mutant group	
	Any	≥Grade 3	Any	≥Grade 3
MDR1 2677	37% (11/30)	13% (4/30)	61% (11/18)	39% (7/18)
MDR1 3535	45% (15/33)	27% (9/33)	47% (7/15)	13% (2/15)
BCRP 421	47% (22/47)	23% (11/47)	0% (0/1)	0% (0/1)
RRMI(-)524	45% (20/44)	25% (11/44)	33% (2/6)	0% (0/6)
RRMI(-)37	43% (20/46)	24% (11/46)	100% (2/2)	0% (0/2)
CDA 208	46% (21/46)	46% (21/46)	50% (1/2)	0% (0/2)

DISCUSSION

For an administered anticancer drug to exert its effect, the drug, after its incorporation into the cell needs to be activated by a drug-metabolizing enzyme. The activated anticancer drug permeates the cell membrane and is released extracellularly. The cell membrane permeation of drugs was previously thought to be the result of simple diffusion. However, recent years in molecular biology have led to the recognition of the importance of cell membrane-localized proteins, called transporters, in drug transport across biological membranes. For example, the function of P-glycoprotein at the blood-brain barrier has been most clearly delineated. It was reported that, in mice deficient in mdr1, the gene encoding P-glycoprotein, some drugs concentrated several tens of times (3). Subsequent advances in the study of drug transporters led to the demonstration of the association between drug sensitivity and the SNP variants of drug transporter genes. Many MDR1 variants were identified before surgery. Among them, 2677G>T, A (exon21) and 3435C>T (exon26) have received attention. In the comparison of the disposition of orally administered digoxin among wild-type homozygotes (2677G/3435C), mutant heterozygotes (2677G/3435C and 2677T/3435T), and mutant homozygotes (2677T/3435T), the ability of cells to transport digoxin was lower in the mutant homozygotes (6). Analysis of the disposition of GEM and paclitaxel showed that their clearance tended to be lower in mutant homozygotes for 2677G>T, A and 3435C>T (however, the 2677G>T, A genotype was identified in only 2 patients each who had received GEM and paclitaxel, respectively, and the 3435C>T genotype in only 1 patient each who had received GEM and paclitaxel, respectively (7). In the present study, 2766G>T, A and 3435C>T genotype of MDR1 were examined. As expected, GEM remained at high concentrations in mutant homozygotes (the mutant group), resulting in a high incidence of side effects. In contrast, G3 or higher side effects were prominent in the normal group of MDR1. On the other hand, reduced drug efflux from the cells resulting in high intracellular GEM concentrations was expected to enhance the effect of GEM. As a result, in the mutant group of MDR1 2677, G3 or higher side effects were prominent and the disease free or overall survival tended to be longer. However, studies have reported different effects of MDR1 SNP genotypes on pharmacokinetics. These discrepancies reflect the specificity of the substrate drugs used and differences in the position of SNP sites in MDR, as well as the difficulty in evaluating P-glycoprotein functioning in a specified transport direction in many locations.

Like P-glycoprotein, BCRP belongs to ABC transporters and many variants have been detected (22). There are three variants involving amino acid substitutions: 34G>A (12Val>Met), 376C>T (126Gln>stop codon) and 421C>A (141Gln>Lys), among which 421C>A has received attention. In a clinical study of patients treated with diflomotecan (a derivative of the anticancer drug camptothecin), the decreased expression of BCRP protein was observed in patients with BCRP variants and their blood diflomotecan levels fluctuated within a high range (21). The reported distribution of variant BCRP 421 genotypes in a Japanese population was 53, 38 and 6.6% for C/C, C/A and A/A, respectively (8), which differed from the present study in that C/C, C/A and A/A accounted for 98, 2 and 0%, respectively. Thus, we could not proceed with further analysis of patients homozygous for a mutant allele.

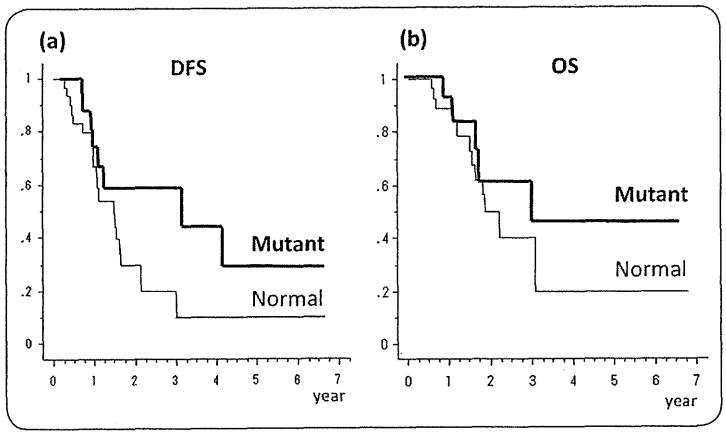

FIGURE 1. (a) Disease-free survival of 18 patients in the mutant group of MDR1 2677 (thick lines) and that of 30 patients in the normal group of MDR1 2677 (thin lines). (b) Those of overall survival.

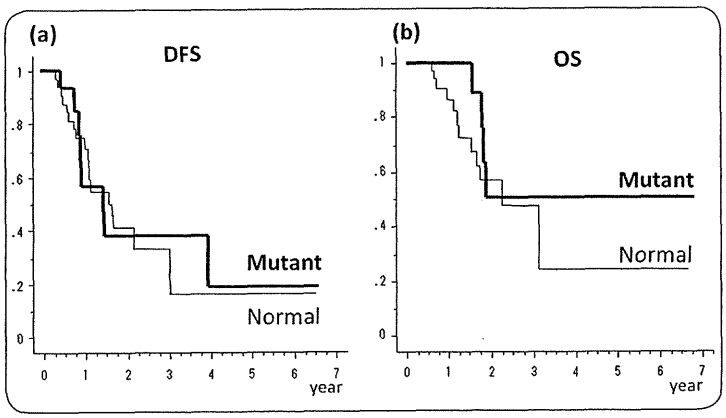

FIGURE 2. (a) Disease-free survival of 15 patients in the mutant group of MDR1 3435 (thick lines) and that of 33 patients in the normal group of MDR1 3435 (thin lines). (b) Those of overall survival.

GEM is a prodrug which is phosphorylated *in vivo* by dCyd kinase to its active form, dFdCTP. On the other hand, dCTP, which competes with dFdCTP in the cells, is also activated by dCyd kinase (10). When the concentration of dCTP is lowered, the activity of dCyd kinase is increased through a feedback mechanism and the metabolism proceeds from GEM to dFdCTP, resulting in the maintenance of a high intracellular level of the active form of GEM. It is believed that the inactivation of dCyd kinase due to any cause results in a decreased level of the active form of GEM, leading to the acquisition of GEM resistance (10,11). Since ribonucleotide reductase (RR) increases the dCTP pool in the cells, the elevation of its activity results in an increase in the intracellular concentration of dCTP with a relative decrease in the level of the active form of GEM (GEM resistance). Conversely, the active form of GEM inhibits RR activity, resulting in a decrease in the intracellular dCTP level with a relative increase in the intracellular GEM level, leading to increased GEM toxicity. In other words, severe side effects occur, but GEM sensitivity can be induced (13). Ribonucleotide reductase activity varies among individuals. The C>A allele (expression rate, 49%) in the promoter region RRM1(-)37 of the RR-encoding gene RRM1 and the T>C allele (expression rate, 59%) in the promoter region RRM1(-)524 are associated with increased RR activity. Thus, in patients with RRM1 SNP mutations, RR activity is decreased and the dCTP pool is decreased in the cells, resulting in a relative increase in the intracellular toxicity of the active form of GEM, leading to the acquisition of GEM sensitivity (14). In the present study, G3 or higher side effects were observed in 24-25% of the patients in the normal group (large RR activity) but in none of those in the mutant group (small RR activity). These results were compatible to our expectation that the intracellular toxicity of the active form of GEM would decrease in the RRM1 normal group.

The pro-drug GEM is inactivated through deamination by CDA to 2',2'-difluorodeoxyuridine (dFdU). It has been reported that G-to-A mutation at the CDA 208 exon3 SNP site results in an alanine-to-threonine substitution (allele expression rate, 4.3%), leading to reduced enzyme activity. With reduced CDA activity, the degradation of pro-GEM to dFdU does not proceed, resulting in the maintenance of a relatively high concentration of the active form of GEM. In the present study, the incidences of G3 or higher side effects in the normal and mutant groups were 24 and 0%, respectively. These results were consistent with the prediction that the relative intracellular concentration of the active form of GEM would decrease in the CDA variant group. The RRM1(-)524, RRM1(-)37 and CDA variant groups comprised only 6, 2 and 2 of the 48 patients, respectively. Their numbers were too small to analyze statistically but their survival times did not appear to differ from those in their respective normal groups. Various studies reported different results on the correlation between the level of RRM1 expression and the prognosis. Bleper *et al.* (13) reported that RRM1 overexpression in lung cancer patients was associated with a favorable prognosis. On the other hand, Rosell *et al.* (15) reported that RR gene over expression provides a dNTP pool, leading to the acquisition of GEM resistance, resulting in a poor prognosis. Yoshimori *et al.* (19) also showed that 208G>A was associated with a 5-fold increase in the AUC. They also analyzed SNP at RRM1 42, 33, (-) 27 and CDA 111 sites (different from the sites analyzed in the present study) in 47 GEM-administered, unresected or stump-positive patients with pancreatic cancer but found no association between SNP genotypes and the prognosis (20). In the present study, we classified wild-type homozygotes and mutant heterozygotes as normal and mutant homozygotes as mutant. In contrast, Okazaki *et al.* (20) classified only wild-type homozygotes as normal and compared them with mutant hetero- plus homozygotes. No evidence has been presented as to which of hetero- and homozygous SNP mutants should be classified as mutant. To investigate to what extent SNP allele differences reflect the activity of intracellular enzymes, further studies at the cellular level are needed.

It is difficult to explain the acquisition of an anticancer drug-resistant and the prediction of an adverse event only by SNP of MDR1 and so on; however, MDR1 2677 must be the most important SNP when we review the property of the chemotherapy against carcinoma.

CONCLUSIONS

In this study, patients who had undergone adjuvant chemotherapy with GEM after pancreatic cancer surgery were studied. This therapy has been established as the standard therapy and chemotherapy regimens do not significantly differ between centers. Since the patients had undergone a highly invasive procedure, like pancreatic cancer surgery, the pathological conditions in which the occurrence of cancer drug side effects was most undesirable, were studied. Among the six SNPs, we were able to show that MDR1 2677 mutant homozygotes were prone

to develop side effects but might achieve prolonged survival. For the practical application of tailor-made medicine, we investigated genomic information and drug responsiveness. We consider that the promotion of inexpensive techniques for SNP analysis (PCR-RFLP in this study) facilitates the sharing of pharmacogenomic information on individual patients through comprehensive genomic analysis, leading to the establishment of individually optimized cancer chemotherapy.

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Original Paper

Single Nucleotide Polymorphism of Multidrug-Resistance 1 and Anti-Multidrug-Resistance 1 Single Chain Antibody Treatment for the Pancreatic Cancer Cell Line

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Key Words: MDR1; Antibody; SNP; Pancreatic cancer.

Abbreviations: Single Nucleotide Polymorphism (SNP); Multidrug-Resistance 1 (MDR1); ATP Binding Cassette (ABC); Single Chain Antibody (scAb); Single Chain Variable Fragment (scFv); Polymerase Chain Reaction (PCR); Variable Region of Light Chain (VL); Variable Region Of Heavy Chain (VH).

ABSTRACT

Background/Aims: Anticancer drugs are essential to pancreatic cancer therapy. The multidrug-resistance 1 (MDR1) gene codes for one of the ATP binding cassette (ABC) transporters. The neutralizing antibody of MDR1 reduces the activity of MDR1 and may add to the sensitivity of anti-cancer drugs. We investigated the relationship of the single nucleotide polymorphisms (SNPs), 2677G and 3435C, in the MDR1 gene and the effect of the anti-MDR1 single chain antibody (scAb) using pancreatic cancer cell lines. **Methodology:** We exposed the pancreatic cancer cell lines, AsCP-1, Panc-1, BxPC-3, MIAPaCa-2 and QGP-1 to 0.1-1,000μg/mL of 5-FU for 72h and calculated the cytotoxic reactions. Combined therapy with an established anti-MDR1 neutralizing scAb and 10μg/mL of 5-FU was also performed. **Results:** AsCP-1 contained wild types of MDR1 2677G and 3435C, and showed the most 5-FU resistance. The anticancer effect of AsPC-1 increased with anti-MDR1 scAb, but the effect was not significant compared with other cell lines. **Conclusions:** The cells with the wild type SNPs of MDR1 showed drug resistance, but we were not able to confirm a remarkable effect of the anti-MDR1 antibody.

INTRODUCTION

Pancreatic cancer has a poor prognosis. Furthermore, it recurs at a high rate regardless of whether it is surgically removed. Therefore, despite ablative therapy, chemotherapy is required (1). The effects and side effects of the anticancer drug show individual differences, and severe adverse events reduce the quality of life of the patient. It is known that cell membrane-localized ATP binding cassette (ABC) transporters are involved in the development of adverse events and cancer drug resistance (2). Single nucleotide polymorphisms (SNPs) of the ABC transporter genes can be associated with reduced transporter activity, leading to excessive adverse events. Among the SNPs of the ABC transporter genes, those of the multidrug-resistance 1 (MDR1) genes encoding P-glycoprotein have been most widely studied (3-6). P-glycoprotein is expressed in organs involved to the transmission of food materials, such as the liver and alimentary canal (7). Therefore, anticancer drugs tend to be less effective for cancers derived from the liver or gastrointestinal tissue. Furthermore, P-glycoprotein contributes to the natural resistance to anticancer drugs (2,3). In recent years, genetic SNPs have been shown to cause the ABC transporter to slow down, and might also promote more severe side effects. In contrast, the decrease in transporter activity induces maintenance of the intracellular drug level (8,9). Many MDR1 SNPs have been identified before surgery. Among these, 2677G→T(A) (exon 21) and 3435C→T (exon 26) have received attention. Because MDR1 is activated in wild type homozygotes (2677G/3435C), drug resistance is high, and, in contrast, a drug effect is found in mutant homozygotes (2677T(A)/3435T) (10). Therefore, we investigated the relationship between SNP of MDR1 and the effect of the antiMDR1 antibody treatment using the pancreatic cancer cell line.

METHODOLOGY

Cell lines and assays

The human pancreatic ductal carcinoma cell lines AsCP-1, Panc-1, BxPC-3 and MIAPaCa-2 were purchased from the American Type Culture Collection (Manassas, VA), and the pancreatic neuroendocrine cell carcinoma cell line (QGP-1) was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). Cells were cultured at 37°C in RPMI-1640 (GIBCO, Life Technologies Japan Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO_2. For cell viability assay, cells were cultured in 96-well microplates for 24h at a volume of 100μL (10,000 cells/well). To evaluate the sensitivity of cancer cells to 5-FU, a suspension of these cells in a serum-free medium was placed in 96-well plates at a

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TABLE 1. The sequence of anti-MDR1 single chain antibody constructed with the genes of anti-MDR1 scFv was obtained from the antibody showing MDR1 protein-neutralizing activity including linker of (Gly3 Ser Ala3) x4 between the variable region and the light chain (VL) and the heavy chain (VH).

VH	GAGGTGAAGCTGGTGGAGTCTGGAGGAGGCTTAGTGAAGTTTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCTG-GATTCACTCTCAGTAGCTATTACATGTCTTGGGTTCGCCAGAGTCCAGAGAAGAGGCTGGAGTTGGTCGCAGTTATTAATAG-TAATGGTGGCAGCACCTACTATCCAGACACTGTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACTTTG-TACCTGCAAATGAGCAGTCTGAAGTCTGAGGACACAGCCTTGTATTACTGTGCAAGACCCTTCTACTATAGTAACTCCCC-GTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA
Linker	GGCGGAGGCGGATCCGGTGGTGGCGGATCTGGAGGTGGCGGAAGC
VL	GATGTTTTGATGACCCAGACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGCAGATCTAGTCAGAG-CATTGTACATAGGACTGGAAACACCTATTTAGAATGGTACCTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTA-CAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAG-CAGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGCTTTCAAGGTTCACATGTTCCGTACACGTTCTCGAG TGA

volume of 90μL (2,000 cells/well) at 37°C. When the cells became adherent to the plate 24h later, 5-FU was added at levels of 0.1-1,000μg/mL and incubated for a further 72h. Cell survival was measured using a WST-1 Cell Counting kit (Wako Co. Ltd., Osaka, Japan).

Evaluation of single nucleotide polymorphism of MDR1 2677 and MDR1 3435

Genomic DNA was isolated from peripheral lymphocytes from venous blood (2mL) of patients with using QIAamp DNA Blood Kit (Qiagen Inc., Valencia, CA, USA). Target site of DNA was amplified by PCR which was performed a total volume of 50μL in the presence of 100μg of cDNA, SuperMix (Invitrogen Corp., Carlsbad, CA, USA) and 0.25μM each primers of the forward primer 5'-agaagcatgagttgtgaaga-3' and the reverse primer 5'-gcatagtaagcagtagggag-3' for MDR1 2677, and the forward primer 5'-tgatggcaaagaaataaagcg-3' and the reverse primer 5'-tgactcgatgaaggcatgtatgt-3' for MDR1 3435. After an initial denaturation, 35 cycles of 10s at 98°C, 30s at 55°C and 1min at 72°C, 5min at 68°C and a final extension period were carried out. The mutations at MDR1 2677 and MDR1 3435 were confirmed by a DNA sequences directly on a 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA) using a Big-Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems). The sequencing primers were those used in the polymerase chain reaction (PCR) amplification.

Construction of anti-MDR1 single chain antibody

We developed two single chain antibodies (scAb) using the following methods. The amino acid sequence of the anti-MDR1 single chain antibody constructed with the complementarity-determining region of the mouse and humanized framework region (single chain variable fragment: scFv) was obtained from the antibody showing MDR1 protein-neutralizing activity engineered by Niv *et al.* (11). These genes of anti-MDR1 scFv including linker of (Gly3 Ser Ala3) x4 between the variable region of the light chain (VL) and that of the heavy chain (VH) were synthesized and cloned as independent segments by employing a combination of oligonucleotide synthesis and PCR. They were inserted into the expression cassette of a plasmid showing unique restriction sites which permitted the assembly of genes including (5' to 3') the cytomegalovirus promoter/enhancer, an optimal Kozak sequence (GCCCCACC), the human Igk-chain secretion signal sequence, a sequence encoding a human constant k-domain, 6 his tag +myc tag to aid in identification, and an SV40 polyadenylation signal (anti-MDR1 scAb) (**Figure 1A**). The amino acid sequence showed specificity for MDR1-positive cell-binding activity (**Figure 1B**). The number of bases that encoded anti-MDR1 scAb were 834 bases. Non-specific scAb was constructed with non-specific scFv instead of anti-MDR1 scFv as a control scAb. These constructs were sequenced to demonstrate their fidelity (**Table 1**). To have anti-MDR1 scAb protein as a reagent for assessment of antibodies against MDR1, the anti-MDR1 scAb gene was also inserted into the T7 promoter driven prokaryotic expression plasmid pRSET (Invitrogen, Carlsbad, CA, USA). This plasmid was transformed into the BL21. The anti-MDR1 scAb protein was purified by passage through a Ni21 column (ProBond kit; Invitrogen) under denaturing conditions. The purity of the protein was confirmed by SDS–polyacrylamide gel electrophoresis and its identity was confirmed by western analysis with an anti-Xpress-HRP antibody (Invitrogen) (**Figure 2**).

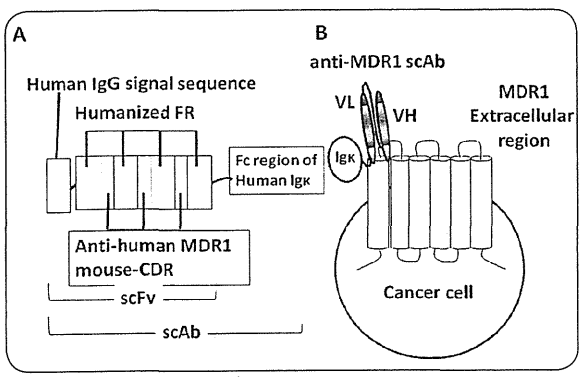

FIGURE 1. (**A**) Structure of anti-MDR1 scAb. Each VH and VL of anti-MDR1 (scFv), and the human constant are connected. VL: variable region of light chain, VH: variable region of heavy chain. (**B**) Schematic diagram of the anti-MDR1 scAb. Anti-MDR1 scAb shows specificity to extracellular domain of MDR1 on the membrane of cancer cells as a target antigen.

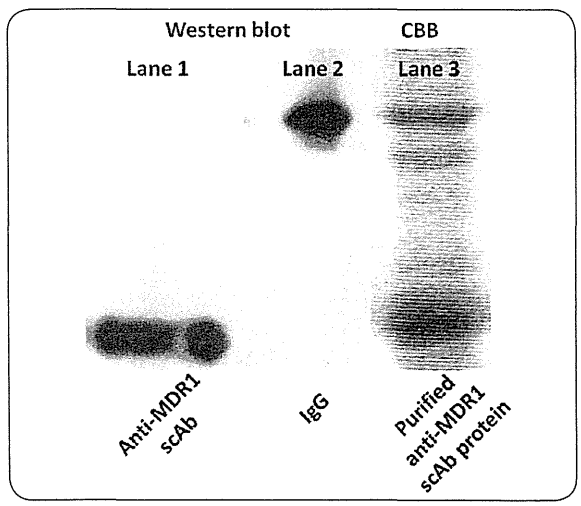

FIGURE 2. Lane 1: the expression of anti-MDR1 scAb is detected by an anti-human k-chain (C6) antibody and lane 2: human IgG as a control by western blot. Lane 3: the purified anti-MDR1 scAb protein by CBB stain.

Statistical analysis

Statistical analyses were performed using StatView (Abacus Concepts Inc., Berkeley, CA, USA). The Mann-Whitney U test was used for WST-1 assays. A two-sided p value of <0.05 was considered to represent a statistically significant difference.

RESULTS

In AsPC-1, both MDR1 2677G and 3435C was a homogenous wild type. In Panc-1, MDR1 2677 was heterogeneous mutant showing G→G/T and MDR1 3435 was homogenous mutant showing C→T. As for BxPC-3, MIAPaCa-2 and QGP-1, MDR1 2677 was homogenous mutant of G→T, and MDR1 3435 was a homogenous mutant of C→T (**Figure 3A,B).**

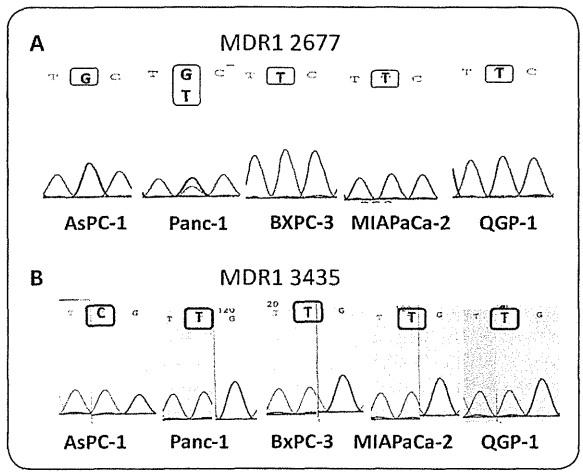

FIGURE 3. The direct DNA sequencing of the PCR product of MDR1 2677 were shown at (A), and those of MDR1. 3435 were shown at (B).

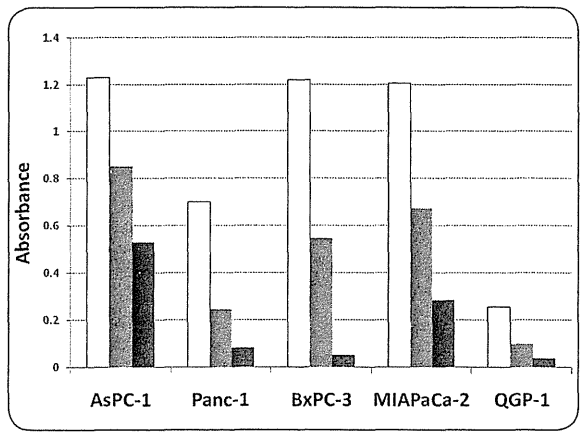

FIGURE 4. The value of cell viability (absorbance of WST-1 assay) of each cancer cells treated with 10^{-1}μg/mL (white bar), 10μg/mL (gray bar) and 1mg/mL of 5FU (black bar).

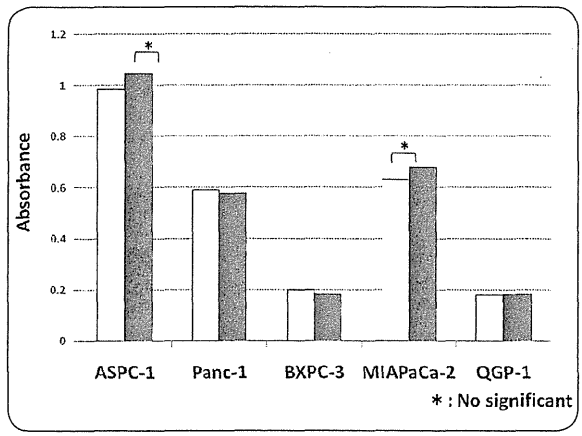

FIGURE 5. The value of WST-1 assay of each cancer cells treated with anti-MDR1 scAb plus 5FU (10μg/mL) is shown as a white bar, and that with non-specific scAb plus 5FU (10μg/mL) is shown as a gray bar.

None of the cell lines except AsPC-1 showed any cytotoxic reaction at 0.1μg/mL of 5-FU, and all cell lines perished at 1,000μg/mL of 5-FU. As for AsPC-1, the cytotoxic reaction was nearly half at 1,000μg/mL of 5-FU, but AsPC-1 clearly showed 5-FU resistance compared with the other cell lines (**Figure 4**).

The values of the cell survival WST-1 assay of AsPC-1, Panc-1, BxPC-3, MIAPaCa-2 and QGP-1 cells were 0.987, 0.590, 0.200, 0.630 and 0.180 respectively, by adding an anti-MDR1 scAb to the culture medium at 10μg/mL of 5-FU. The values obtained by adding a non-specific scAb were 1.047, 0.576, 0.182, 0.688 and 0.182, respectively. There was a therapeutic effect (decrease of the value of WST-1 assay) in anti-MDR1 scAb in AsPC-1 and MIAPaCa-2 which showed 5-FU resistance (**Figure 5**).

DISCUSSION

For an anticancer drug to be able to exert its effect, it is necessary for this medicine to interact with a drug-metabolizing enzyme after its incorporation into the cell. The activated anticancer drug permeates the cell membrane and is excreted extracellularly. It was previously thought that cell membrane penetration of the medicine was the result of single diffusions. In recent years, the importance of proteins localized on the cell membrane, called transporters, has been recognized. In mice deficient in the mdr1 gene encoding P-glycoprotein, some drugs were concentrated several tens of times (12). Subsequent advances in the study of drug transporters has led to the demonstration of the association between drug sensitivity and SNP variants of the MDR1 gene in particular 2677G→T(A) in exon21 and 3435C→T in exon26 (9). In the comparison of the disposition of orally administered digoxin among 2677G/3435C (wild type-wild type), 2677G/3435T and 2677T/3435C (wild type-mutant type, mutant type-wild type), 2677T/3435T (mutant type-mutant type), the ability of cells to transport digoxin was lower in the mutant homozygotes (10). Analysis of the disposition of gemcitabine, a standard drug for pancreatic cancer, and paclitaxel showed that their clearance tended to be lower in mutant homozygotes for 2677G→T(A) and 3435C→T. In the pancreatic cancer cell lines used in this study, only AsPC-1 showed 2677G and 3435C (a wild type combination), Panc-1 showed a heteromutation of 2677G→G/T and homogeneous mutation of 3435C→T, and the other cell lines were mutant combinations of MDR1 2677G→T and 3435C→T. In AsPC-1, which is 2677G/3435C (a wild type combination), MDR1 activity was high and the density of 5-FU was not maintained; therefore, AsPC-1 showed drug resistance. In contrast, in BxPC-3 and QGP-1, the activity of MDR1 2677T/3435T (a mutant type combination) was low and intracellular levels of 5-FU were expected to be maintained by the transport ability of the drug. The results in this study were generally in accordance with the predicted results described above. Furthermore, in AsPC-1, recovery of remarkable chemical sensitivity was expected by anti-MDR1 antibody administration, and some recovery was achieved; therefore, there was not a significant difference from the other cell lines. These discrepancies reflect the specificity of the substrate drugs used and differences in the positions of SNP sites in MDR1, as well as the difficulty in evaluating P-glycoprotein functioning in a specified transport direction in many locations. In the future, an examination in the cell unit is necessary to determine how a difference in the SNP affects the quantity of intracellular drug concentration.

CONCLUSIONS

We confirmed that there was drug resistance in AsPC-1 containing wild type SNP of MDR1. However, anti-MDR1 antibody treatment of AsPC-1 was expected to induce a remarkable recovery of chemical sensitivity, but little recovery was obtained. In conclusion, a specific effect of the anti-MDR1 antibody was not confirmed in this examination.

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The clinical significance of SWI/SNF complex in pancreatic cancer

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Abstract. Chromatin remodeling factors have been the subject of great interest in oncology. However, little is known about their role in pancreatic cancer. The objective of this study was to clarify the clinical significance of the SWItch/sucrose non-fermentable (SWI/SNF) complex in patients with pancreatic cancer. A total of 68 patients with pancreatic cancer who underwent R0, 1 resection were enrolled. Cancer tissues were processed to tissue microarray, then stained immunohistochemically by using antibody of SWI/SNF components; BRM, BRG1, BAF250a, BAF180 and BAF47. The correlation of expression levels and clinicopathological outcomes were analyzed, followed by the multivariate analysis of prognostic factors for overall survival. The expression levels of the SWI/SNF components were categorized as low or high according to the median value of Histoscore. Statistical analysis revealed that BRM expression was related to tumor size, T factor, M factor, lymphatic invasion and stage BRG1 expression to histology and stage BAF180 expression to tumor size and BAF47 expression to lymphatic invasion, respectively. Multivariate Cox proportional hazard analysis showed that high BRM and low BAF180 expression levels were independent predictors of worse survival in patients with pancreatic cancer. High BRM, and low BAF180 were also independent prognostic factors for poor survival in the subgroup with adjuvant gemcitabine. These results suggest that the specific cofactors of SWI/SNF chromatin remodeling complex certainly have roles in pancreatic cancer. High BRM, and low BAF180 are useful biomarkers for poor prognosis in pancreatic cancer.

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Key words: pancreatic cancer, the SWItch/sucrose non-fermentable complex, prognostic factor

Introduction

Pancreatic cancer remains a leading cause of cancer deaths in the advanced nation (1,2). The overall 5-year survival rate is reported to be less than 5% (3). A reliable and clinically relevant prognostic biomarker which can stratify the disease is needed for developing new strategies.

It is a known fact that chromatin, highly condensed and dynamically structured, can be temporally rearranged so that specific genes can be expressed or repressed (4). Studies have shown that modification of chromatin structure is an essential step in gene regulation primarily mediated by chromatin remodeling proteins. Among these proteins, histone is known to play a dynamic role in the regulation of transcription (5-7). Often, transcription is also regulated by other cofactors, and the balance of chromatin remodeling activities may be crucial to ensure accurate responses to developmental or environmental cues and to prevent the transition of normal cells into cancer cells (8).

The SWItch/sucrose non-fermentable (SWI/SNF) complex is a major complex of adenosine triphosphate (ATP)-dependent chromatin remodeling factors and controls the transcriptional activity of a variety of genes involved in cellular growth and transformation by altering chromatin structure (9-13). SWI/SNF complex, originally identified in yeast, is composed of more than 10 characterized subunits (14,15) and human SWI/SNF complexes contain one of the two core ATPase subunits, BRM or BRG1 (13,16-18). Growing genetic and molecular evidence indicates that specific subunits of the SWI/SNF complex can act as tumor suppressors (6,19). However, there is no report on the relationship between SWI/SNF components expression and the clinical significance of pancreatic cancer. In this study, we investigated the expression levels of SWI/SNF components to clarify the clinical impact of SWI/SNF complex on pancreatic cancer.

Materials and methods

Patients and samples. The surgical specimens of pancreatic cancer tissue obtained from 68 patients were evaluated. All of the patients had undergone macroscopically curative resection (R0, 1) at Kanagawa Cancer Center between July 2006

and April 2010. The clinicopathological characteristics of these patients are shown in Table I. In all cases, archival hematoxylin and eosin-stained (H&E) slides of the primary tumor were retrieved and reviewed to confirm the pathological features as well as to select suitable tissue blocks for immunohistochemical analysis. Informed consent was obtained from each patient. The Ethics Committees of the Kanagawa Cancer Center approved the protocol before initiation of the study. We declare no conflicts of interest.

Tissue microarrays and immunohistochemistry. Microarrays consisting of cores, each measuring 2 mm in diameter, were prepared from formalin-fixed paraffin-embedded tissue blocks of surgically removed primary tumors. Each tissue core of the primary tumor was sampled.

Immunohistochemical staining was performed using commercially available polyclonal rabbit, or mouse antibodies raised against BRM (Abcam Inc., Cambridge, MA), BRG1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), BAF250a (Santa Cruz Biotechnology Inc.), BAF180 (Sigma-Aldrich Inc., St. Louis, MO), BAF47 (Santa Cruz Biotechnology Inc.). Tissue microarray blocks were sectioned at a thickness of 4 μm and mounted on pre-coated glass slides. The sections were de-paraffinized through a graded series of xylene and rehydrated through a graded series of alcohol to distilled water. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature. The sections were placed in a 95˚C solution of 0.01 M sodium citrate buffer (pH 6.0) for 40 min for antigen retrieval. Normal goat serum (5%) was then applied for 15 min to block any non-specific protein binding sites. Primary polyclonal antibodies were applied for 1 h at room temperature at the following dilutions: anti-BRM at 1:250, anti-BRG1 at 1:200, anti-BAF250a at 1:100, anti-BAF180 at 1:90 and BAF47 at 1:300. Immunoreactive proteins were detected using the Simple Stain MAX-PO (Multi).

All sections were counterstained with Mayer's hematoxylin, and negative controls were included in each staining sequence. The intensity and global level of staining were scored semi-quantitatively for each tissue microarray by an investigator blinded to all of the clinicopathological variables. The global level of staining refers to the percentage of tumor cells that stained positively for an antibody within each tissue microarray at x200 magnification using a light microscope.

Scoring of immunohistochemical reactivity. Immunohistochemical scoring was completed using the modified Histoscore (H-score) (20), which involves a semiquantitative assessment of both the intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong using adjacent normal mucosa as the median) and the percentage of positive cells (Fig. 1). The range of possible scores was from 0 to 300. Expression level of each component was categorized as low or high according to the median value of H-score.

Statistical analysis. The relationships between the expression level and the clinicopathological factors were evaluated with the χ^2 test. The postoperative survival rate from the day of primary tumor resection was analyzed using the Kaplan-Meier method and any differences in the survival rates were assessed with the log-rank test. A Cox proportional-hazard model was used for the multivariate analyses. Differences were considered significant when $P<0.05$. The statistical analysis was performed using the PASW Statistics 18 (SPSS, Inc., Chicago, IL).

Table I. The clinicopathological characteristics of all patients.

Clinicopathological characteristics	No. of patients (n=68)
Age	
<65	30
≥65	38
Sex	
Male	36
Female	32
Tumor location in pancreas	
Head	46
Body/tail	22
Tumor size (cm)	
<4	29
≥4	39
Histological type	
Well/mod	32
Poor	36
T	
T1-3	38
T4	30
N	
N0	17
N1	51
M	
M0	53
M1	15
Curability of surgery	
R0	43
R1	25
Stage	
0-III	53
IV	15
Adjuvant gemcitabine	
Yes	42
No	26

Well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.

Results

Relation of SWI/SNF component expression to clinicopathological features. The distribution of H-score is showed in Fig. 2.

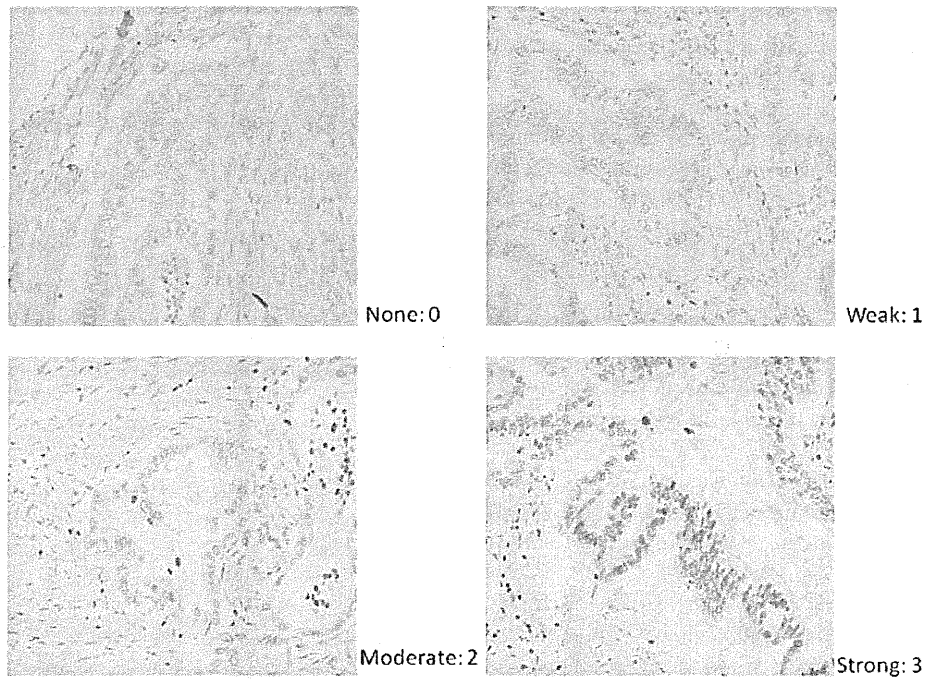

Figure 1. Histoscore (H-score) was calculated by a semi-quantitative assessment of both the intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong using adjacent normal mucosa as the median) and the percentage of positive cells. The range of possible scores was from 0 to 300. Expression level of each component was categorized as low or high according to the median value of the H-score.

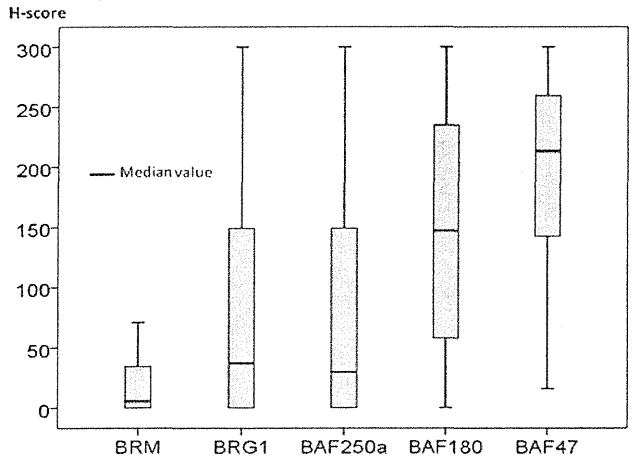

Figure 2. The distribution of the H-score is shown in the box plot. The horizontal bar shows the median value of each score.

Expression level of the SWI/SNF components was categorized as low or high according to the median value of the H-score. Relations between the expression levels of each component and clinicopathological features were then examined. Factors implicating significant relations were tumor size, T factor, M factor, lymphatic invasion, and stage in BRM, histology and stage in BRG1, tumor size in BAF180, lymphatic invasion in BAF47, respectively (Table II).

Analysis of prognostic factors in all patients. Univariate Cox regression analysis for overall survival in all patients showed that age, tumor size, histological type, M factor, curability of the surgery, and expression level of BRM as well as BAF180 were significant predictors (Table III). On multivariate Cox proportional hazard analysis, histology, expression level of BRM and BAF180 were significant independent predictors of overall survival in patients with pancreatic cancer (Table IV).

Comparison of survival by the status of BRM and BAF180. The 5-year survival rate of high BRM patients was 9.8%, which was significantly worse than that of low BRM patients (43.8%) (Fig. 3). Also, the 5-year survival rate of low BAF180 (8.1%) was significantly worse than that of high BAF180 patients (40.8%) (Fig. 3).

Hazard analysis of SWI/SNF components in the patients treated with adjuvant gemcitabine. Multivariate analysis (Table V) and survival analysis (Fig. 4) showed that BRM-high and BAF180-low were independent prognostic factors for overall survival in the patients treated with adjuvant gemcitabine.

Discussion

Chromatin remodeling factors have been the subject of great interest in oncology. However, little is known about their role in pancreatic cancer.

The SWI/SNF complexes are large, multi-subunit complexes containing 10 or more subunits, serving as a master switch that directs and limits the execution of specific cellular programs, such as differentiation and growth control (21). Each complex has one of the two different ATPase as core motor; BRM or BRG1, and subunits which are referred to as BAFs (BRM- or BRG1-associated factors). The BRM-containing complex is termed BRM/BAF. The BRG1-containing complexes are

Table II. Relation of SWI/SNF component expression to clinicopathological factors.

	BRM			BRG1			BAF250a			BAF180			BAF47		
Factors	Low	High	p-value	Low	High	p-value	Low	High	p-value	Low	High	p-value	Low	High	p-value
Age (years)															
<65/≥65	15/19	15/19	1.000	18/19	12/22	0.143	13/21	17/17	0.329	19/15	11/23	0.051	13/21	17/17	0.329
Gender															
Male/female	16/18	16/18	1.000	16/18	16/18	1.000	13/21	19/15	0.145	15/19	17/17	0.627	17/17	15/19	0.627
Tumor size															
<4/≥4 cm	19/15	10/24	0.027	12/22	17/17	0.220	14/20	15/19	0.806	10/24	19/15	0.027	15/19	14/20	0.806
Histology															
Well, mod/poor	18/16	14/20	0.331	11/23	21/13	0.015	14/20	18/16	0.331	13/21	19/15	0.145	15/19	17/17	0.627
T															
T1-3/4	25/9	13/21	0.003	23/11	15/19	0.051	17/17	21/13	0.329	19/15	19/15	1.000	20/14	18/16	0.625
N															
N0/N1	9/25	8/26	0.779	10/24	7/27	0.401	10/24	7/27	0.401	8/26	9/25	0.779	9/25	8/26	0.779
M															
M0/M1	30/4	23/11	0.041	27/7	26/8	0.770	24/10	29/5	0.114	25/9	28/6	0.380	28/6	25/9	0.380
Vessel invasion															
No/yes	12/22	8/26	0.287	11/23	9/25	0.595	7/27	13/21	0.110	10/24	10/24	1.000	8/26	12/22	0.287
Lymphatic invasion															
No/yes	15/19	6/28	0.018	13/21	8/26	0.189	9/25	12/22	0.431	9/25	12/22	0.431	15/19	6/28	0.018
Stage															
0-III/IV	18/16	5/29	0.001	17/17	6/28	0.005	10/24	13/21	0.442	11/23	12/22	0.798	14/20	9/25	0.200
Curability															
R0/R1	25/9	18/16	0.078	23/11	20/14	0.451	20/14	23/11	0.451	21/13	22/12	0.801	20/14	23/11	0.451

Well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; inv, invasion.

Table III. Univariate analysis for overall survival in pancreatic cancer.

Factors	HR (95% CI)	p-value
Age (years)		0.035
<65	1.0	
≥65	0.533 (0.293-0.967)	
Sex		0.632
Male	1.0	
Female	0.865 (0.478-1.565)	
Tumor size (cm)		0.035
<4	1.0	
≥4	1.979 (1.048-3.739)	
Histology		0.002
Well/mod	1.0	
Poor	2.744 (1.429-5.271)	
T		0.071
T1-3	1.0	
T4	1.733 (0.955-3.146)	
N		0.602
N0	1.0	
N1	1.208 (0.594-2.458)	
M		0.010
M0	1.0	
M1	2.329 (1.222-4.439)	
Curability of surgery		0.020
R0	1.0	
R1	2.068 (1.121-3.815)	
BRM		0.011
Low	1.0	
High	2.225 (1.199-4.129)	
BRG1		0.601
Low	1.0	
High	0.853 (0.471-1.546)	
BAF250a		0.479
Low	1.0	
High	0.807 (0.446-1.461)	
BAF180		0.007
Low	1.0	
High	0.428 (0.231-0.793)	
BAF47		0.226
Low	1.0	
High	0.690 (0.378-1.258)	

HR, hazard ratio; 95% CI, 95% confidence interval; well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.

Table IV. Multivariate analysis for overall survival in pancreatic cancer.

Factors	HR (95% CI)	p-value
Age		0.169
<65	1.0	
≥65	0.633 (0.330-1.214)	
Tumor size (cm)		0.755
<4	1.0	
≥4	1.122 (0.543-2.318)	
Histology		0.011
Well/Mod	1.0	
Poor	2.702 (1.253-5.830)	
M		0.486
M0	1.0	
M1	1.381 (0.557-3.424)	
Curability of surgery		0.076
R0	1.0	
R1	1.981 (0.932-4.214)	
BRM		0.032
Low	1.0	
High	2.144 (1.066-4.311)	
BAF180		0.041
Low	1.0	
High	0.501 (0.258-0.971)	

HR, hazard ratio; 95% CI, 95% confidence interval; well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.

further divided into those that contain the BAF250a (termed BRG1/BAF) or the BAF180 (termed PBAF). These three types of complexes are believed to have different molecular functions (22).

There are several studies reporting that the subunit of SWI/SNF complex was decreased in cancer tissues. They revealed the mutation of *ARID1A*, which codes BAF250a protein, in about half of ovarian clear cell carcinomas (23,24), and *PBRM1*, which codes BAF180, in approximately 40% of renal cell carcinomas (25). Another study identified the SWI/SNF chromatin remodeling complex as tumor suppressor, by mediating retinoblastoma protein (RB)-derived regulation of the cell cycle (22,26,27). However, the roles of these subunits in pancreatic cancers are poorly understood.

In this study, we investigated the expression levels of 5 key subunits; BRM, BRG1, BAF250a, BAF180, which are the key subunits when subdividing complex types, and BAF47. There is established evidence that BAF47 is a tumor suppressor in rhabdoid tumors (28).

In the analysis of expression level and clinicopahological features, high BRM was related to worse clinicopathological features in general, including larger tumor size, T4 disease, other