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Gene Expression Profile Can Predict Pathological Response to Preoperative Chemoradiotherapy in Rectal Cancer

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Abstract. *Background: Preoperative chemoradiotherapy (CRT) has been widely used to improve local control of disease and to preserve the anal sphincter in the treatment of rectal cancer. However, the response to CRT differs among individual tumors. Our purpose of this study was to identify a set of discriminating genes that can be used for characterization and prediction of response to CRT in rectal cancer. Patients and Methods: Seventeen rectal cancer patients who underwent preoperative CRT (40 Gy radiotherapy combined with S-1) were studied. Biopsy specimens were obtained from rectal cancer patients before preoperative CRT and were analyzed by focused DNA microarray (132 genes) and immunohistochemistry. Response to CRT was determined by histopathologic examination of surgically resected specimens and patients were classified as responders (grade 2 or 3) or non-responders (grade 0 or 1). Results: Of the 17 samples, 10 were classified as responders and 7 as non-responders. Seventeen genes were differentially expressed at significant levels ($p<0.05$) between responders and non-responders. All genes showed higher expression in responders as compared with non-responders. The list of discriminating genes included matrix metalloproteinase- (MMP), apoptosis- (nuclear factor kappa light polypeptide gene enhancer in B-cells 2 (NFKB2), transforming growth factor beta 1 (TGFB1)), DNA repair- (topoisomerase 1 (TOP1)), and cell proliferation (integrin, beta 1 (ITGB1))-related genes. In the immunohistochemistry of MMP7, 4 responders were judged as showing overexpression of MMP7. On the other hand, none of the non-responders were judged as showing overexpression of MMP7. Conclusion: Gene expression patterns of diagnostic biopsies can predict pathological response to preoperative CRT with S-1 in rectal cancer.*

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Locoregional recurrence after resection of rectal cancer is difficult to treat and is associated with severe debilitating symptoms. The prognosis after a local recurrence is poor, with a median survival of 12-18 months (1). Preoperative chemoradiotherapy (CRT) has been widely used as a major treatment modality for locally advanced rectal cancer. Clinical trials have demonstrated that preoperative CRT significantly reduces the risk of local recurrence and toxicity compared with postoperative CRT, but with similar survival rates (2-7). Furthermore, the ability to achieve pathologic downstaging or a complete pathologic response after preoperative CRT is correlated with improved survival, decreased local recurrence, and a higher rate of sphincter-preserving surgery (8-10).

Approximately 40-60% of locally advanced rectal cancer patients treated with preoperative CRT achieve some degree of pathologic downstaging. However, response to CRT differs among individual tumors and there is no effective method of predicting which patients will respond to neoadjuvant CRT. Although responders to CRT have many benefits of CRT, non-responders may unfortunately be subject to the risk of toxicity with no apparent gain. It is therefore of the utmost importance to identify factors prior to preoperative CRT that predict whether a patient is likely to be resistant or sensitive to CRT. The ability to analyze predictive markers of CRT at the levels of RNA, DNA, and protein promises to revolutionize our understanding of the disease process, and it is hoped that the era of genomics, transcriptomics, and proteomics will herald new biomarkers of response to CRT. One strategy, using gene array technology, is to compare the relative gene expression profiles of tumors between responders and non-responders to CRT.

In this report, to predict response to CRT with S-1 before preoperative CRT, we examined the gene expression patterns of diagnostic biopsy samples by customized and focused DNA microarray developed to measure molecular markers involved in response to 5-fluorouracil (5-FU) and other anticancer drugs. The purpose of this study was to define the gene expression patterns for prediction of response to CRT with S-1 and establish tailored therapy for rectal cancer.

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Patients and Methods

Patients and tissue samples. For gene expression profiling, rectal cancer samples were obtained from 20 patients approved to receive preoperative CRT from September 2005 to September 2007 at Tokushima University Hospital. The 20 independent rectal tumor samples included 17 for training and 3 for testing the outcome prediction model, respectively. The patient characteristics and response to CRT are summarized in Table I. We obtained study approval from the Ethics Committee at Tokushima University Hospital and each patient gave written informed consent for samples to be used. Biopsy specimens were prospectively collected during colonoscopic examination from rectal cancer before starting preoperative CRT. Parallel tumor specimens were formalin fixed and paraffin embedded for histologic examination and further specimens were used for RNA extraction. Samples were used for RNA extraction when parallel specimens contained at least 70% tumor cells. Samples were snap-frozen immediately in liquid nitrogen and stored at –80°C until RNA extraction was carried out.

All patients received CRT with a total dose of 4,000 cGy of pelvic irradiation; CRT was administered five times weekly, with a daily fraction of 200 cGy, utilizing a four-field technique. Radiation was delivered concomitantly with S-1, a novel oral fluoropyrimidine inhibitor of dihydropyrimidine dehydrogenase which has a potent radiosensitizing property. S-1 was administered on days of radiation. Surgical treatment was performed 6-8 weeks after the completion of preoperative CRT.

Table I. *Patient characteristics and response to CRT.*

	Training (n=17)	Testing (n=3)
Male/female	13:4	2:1
Age (years), median (range)	60 (47-82)	65 (51-77)
Tumor size (cm), median (range)	4.1 (2.5-7.3)	4.6 (3.5-5.8)
Tumor distance from the anal verge (cm), median (range)	2.0 (0-4.0)	2.5 (1-3.0)
Grade of differentiation		
Well/moderately	17	3
Poorly	0	0
Tumor stage		
T3	15	3
T4	2	0
Nodal stage		
N0	13	2
N1	2	1
N2	2	0
Pathological response		
Grade 0	0	0
Grade 1	7	1
Grade 2	8	2
Grade 3	2	0

Customized DNA microarrays. A customized DNA array (132 genes) has been developed to measure simultaneously molecular markers involved in response to 5-FU and other anticancer drugs. They consist of 30 genes related to pyrimidine/purine/folate metabolism (thymidylate synthase, dihydropyrimidine dehydrogenase, *etc.*), 19 genes related to DNA repair (DNA ligase I, uracil-DNA glycosylase, *etc.*), 8 genes related to drug resistance (P-glycoprotein, topoisomerase I, *etc.*), 7 genes related to apoptosis (P53, *etc.*), 24 genes related to proliferation (vascular endothelial growth factor, histone deacetylase 1, *etc.*), 20 genes related to cell cycle (E2F1, cyclin A1, *etc.*), 21 other genes of DNA methylation, cell adhesion and collagen catabolism (DNA (cytosine-5)-methyltransferase 1, CD34, matrix metalloproteinase 1, *etc.*) and 3 housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, beta-actin, 40S ribosomal protein S9). Target DNAs made from the 132 genes were immobilized on a glass plate. Each target DNA (200-600 bp) was designed based on sequence homology analysis to minimize cross-hybridization with other genes, and was practically tested by Northern blot. It was possible to relative determine all genes in a single assay. The basic technology of the customized DNA array is almost the same as that of a Stanford-type cDNA microarray.

Frozen tumor tissues were suspended in RLT Buffer (Qiagen, Hilden, Germany) and homogenized using an MM300 Mixer Mill (F. Kurt Retsch GmbH & Co., Haan, Germany). RNA extraction was performed using an RNeasy mini kit (Qiagen). Total RNA quality was judged from the relative intensities of the 28S and 18S ribosomal RNA bands after agarose gel electrophoresis. Purified total RNA (20 μg) was incubated at 70°C for 5 min and cooled on ice. It was reverse-transcribed with a mixture of specific primers and 200 units of PowerScript reverse transcriptase, and incubated at 42°C for 1.5 h. The cDNA was labeled using Cy5 (Cy5 monofunctional reactive dye, Cat. No. PA25001, GE Healthcare–Amersham Biosciences, Piscataway, NJ, USA), and purified by a Nucleo Spin Extract kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). Labeled cDNA was hybridized in 6× SSC, 0.2% SDS, 0.01 mg/ml Human Cot-1 DNA and 5× Denhalt's solution for 16 h at 60°C for spotted cDNA arrays. The slides were washed in 2× SSC at room temperature, then 2× SSC with 0.2% SDS at 55-65°C twice, and finally 0.05× SSC at room temperature and scanned using an FLA-8000 Scanner (FujiFilm, Tokyo, Japan). Data was analyzed using an Array Gauge (FujiFilm).

Immunohistochemistry. Immunohistochemical staining was performed on 5-μm thick sections obtained from formalin-fixed and paraffin-embedded tissue blocks of biopsy specimens from rectal cancer patients before starting preoperative CRT. Immunostaining was carried out after heat-based antigen retrieval (20 min, 95°C water bath, citrate buffer [pH 6]) using mouse monoclonal antibody against matrix metalloproteinase-7 (MMP7) (Daiichi Fine Chemical, Toyama, Japan; dilution, 1:50). Automated immunohistochemistry was performed using a Dako Autostainer Plus System (DakoCytomation, Carpinteria, CA, USA) with antimouse IgG EnVision Plus detection kit (DakoCytomation) for secondary and tertiary immunoreactions. Reaction products were developed with diaminobenzidine (DAB), according to standard protocols. Sections were considered to demonstrate MMP7 overexpression if more than 50% of the tumor cells were positively stained. Negative control sections with the omission of the primary antibody were included in each run.

Data analysis. To identify genes that were differentially expressed between the two groups, the data sets were assigned to either responders or non-responders. Response to CRT was evaluated by histopathologic examination and DNA microarray was analyzed. Histopathologic examination of surgically resected specimens was based on a semiquantitative classification system as described in

Table II. *Genes differentially expressed between responders and non-responders.*

No.	Gene symbol	Description	Fold change	*P*-value
1	*MMP7*	Matrix metalloproteinase 7	2.63	0.007
2	*MMP14*	Matrix metalloproteinase 14	2.29	0.013
3	*MMP9*	Matrix metalloproteinase 9	1.86	0.013
4	*MMP1*	Matrix metalloproteinase 1	1.85	0.045
5	*ITGA2*	Integrin, alpha 2	1.78	0.045
6	*NFKB2*	NFK light polypeptide gene enhancer in B-cells 2	1.63	0.028
7	*CTSB*	Cathepsin B	1.48	0.005
8	*ITGB1*	Integrin, beta 1	1.48	0.045
9	*MMP16*	Matrix metalloproteinase 16	1.45	0.045
10	*PLAUR*	Plasminogen activator, urokinase receptor	1.40	0.028
11	*DNMT1*	Ribonucleotide reductase M1 polypeptide	1.38	0.028
12	*DNMT1*	DNA (cytosine-5-)-methyltransferase 1	1.38	0.028
13	*UP*	Uridine phosphorylase 1	1.38	0.036
14	*TOP1*	Topoisomerase (DNA) I	1.34	0.022
15	*TGFB1*	Transforming growth factor, beta 1	1.32	0.045
16	*NDKA*	Non-metastatic cells 1, protein	1.30	0.045
17	*NDKB*	Non-metastatic cells 2, protein	1.09	0.045

NFK: Nuclear factor kappa

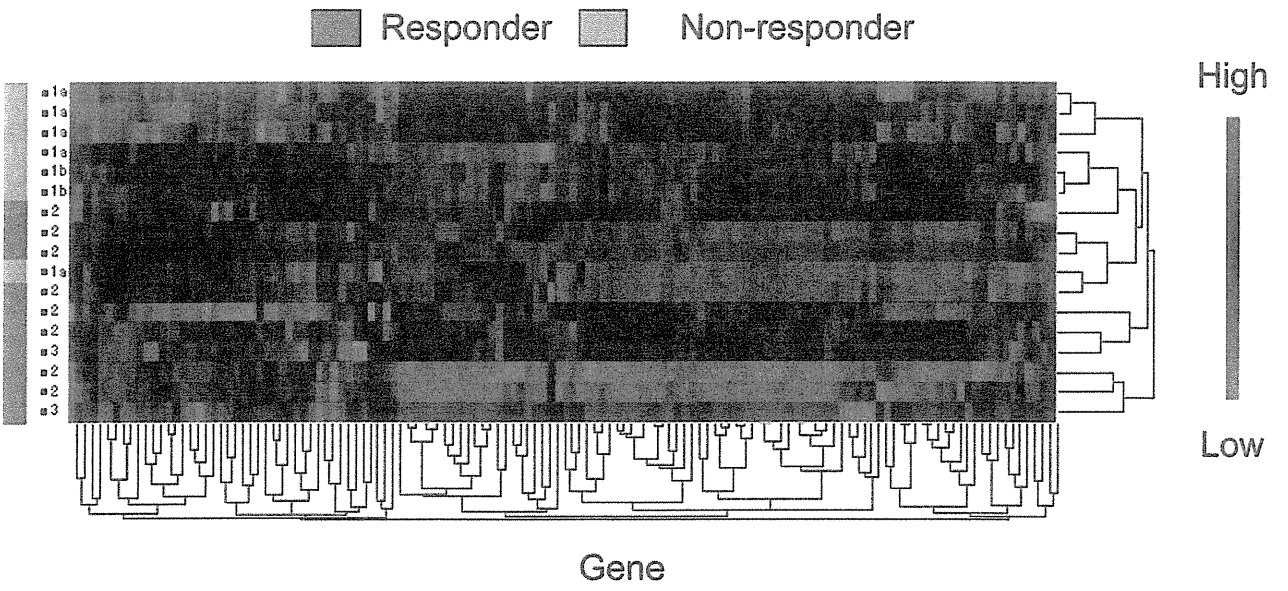

Figure 1. *A hierarchical cluster analysis of 132 genes. Red, Overexpression; green, underexpression. Responders and non-responders were clustered into two distinct groups except for four responder cases.*

detail previously (11). Tumors were classified as "responder" when assigned to the regression grade 2 or 3, and "non-responder" when grade 0 or 1.

Samples from 20 patients were divided into a training set (17 samples) and a testing set (3 samples). Only training samples were used in the DNA microarray analysis to evaluate gene expression. The expression patterns were compared and fold-change value calculated to identify gene markers that can best discriminate between responders and non-responders. Two-dimensional hierarchical clustering was then applied to the log-transformed data with average-linkage clustering with standard correlation as the similarity metric for the discriminating genes that were identified as being differentially expressed between responders and non-responders. Next, using immunohistochemistry in a training set, we evaluated a candidate gene, *MMP7*, detected by DNA microarray analysis as being the most highly overexpressed. Additionally, in a testing set, the gene expression patterns of diagnostic biopsy samples were evaluated by focused DNA microarray before preoperative CRT regarding to the histopathologic examination of surgical specimens.

Table III. *Validation of the gene expression patterns by microarrays.*

Test sample (n=3)	Response to CRT	Overexpressed genes (n)
1	Grade 2	5/17
2	Grade 2	4/17
3	Grade 1	0/17

Statistics. Quantitative data were given as median (range). All statistical analysis was performed using statistical software (JMP 8.0.1., SAS, Cary, NC, USA). A comparison of immunohistochemistry data was performed using Fisher's exact test, as appropriate. The expression patterns in the DNA microarray were compared using unpaired *t*-tests (with Welch's correction for unequal variances). All statistical tests performed were two-sided and declared at the 5% significance level.

Results

Gene expression patterns by microarray in responders and non-responders. Gene expression profiling was established using customized and focused DNA microarray in training samples. There was no significant difference between the training set and the testing set in clinicopathologic factors such as gender, age, histopathologic classification, preoperative tumor stage, response to CRT, and so on. The patient characteristics and response to CRT are summarized in Table I. Among the 17 training samples, 10 were classified as responders and 7 as non-responders, according to the histopathologic examination of surgical specimens. Regarding histopathologic examination of surgically resected specimens, 17 genes were identified that were significantly ($p<0.05$) differentially expressed between responders and non-responders (Table II). All genes showed higher expression in responders as compared with non-responders. The 17 genes were matrix metalloproteinase 7 (*MMP7*), *MMP14, MMP9, MMP1,* integrin, alpha 2 (*ITGA2*), nuclear factor kappa light polypeptide gene enhancer in B-cells 2 (*NFKB2*), cathepsin B (*CTSB*), integrin, beta 1 (*ITGB1*), *MMP16*, plasminogen activator, urokinase receptor (*PLAUR*), ribonucleotide reductase M1 (*RRM1*), DNA (cytosine-5)-methyltransferase 1(*DNMT1*), uridine phosphorylase (UP), topoisomerase 1 (*TOP1*), transforming growth factor, beta 1 (*TGFB1*), nucleoside diphosphate kinase A (*NDKA*), and nucleoside diphosphate kinase B (*NDKB*). Results of a hierarchical cluster analysis of the 132 genes are presented in Figure 1. Responders and non-responders were clustered into two distinct groups except for four responder cases.

Immunohistochemistry of MMP7. MMP7, as a candidate gene, showed the highest fold-change in responders as compared with non-responders in histopathologic examination, and was chosen for validation of DNA microarray data by immunohistochemistry. MMP7 was evaluated by using immunohistochemistry examination in a training set. Four cases out of the responders (n=10) were judged as showing overexpression of MMP7. On the other hand, none of the non-responders (n=7) were judged as showing overexpression of MMP7. There was a tendency for there being a difference in expression of MMP7 between responders and non-responders.

Validation of the gene expression patterns by microarrays. Gene expression profiling (17 genes) was validated using customized and focused DNA microarray in testing samples. Among the three testing samples, two were classified as responders and one as non-responders according to the histopathologic examination of surgical specimens (Table I). One case of the responders showed overexpression of 5 out of 17 genes. Another case in the responders showed overexpression of 4 out of 17 genes. These overexpressed genes included *MMP7* and *TGFB1*. On the other hand, the non-responder case showed no overexpression of any of the genes studied (Table III).

Discussion

Although gene expression patterns have been applied to the outcome prediction of multiple types of cancer, there are few studies to date that have reported the application of DNA array to predict response to CRT using preoperative biopsy tissue samples for rectal cancer. We defined the gene expression patterns for prediction of response to CRT by customized and focused DNA microarray and validated a candidate gene (*MMP7*) by immunohistochemistry.

Regarding DNA microarray, two studies have incorporated microarray analyses to assess gene expression profiles to predict CRT outcome in rectal cancer. In a study by Ghadimi *et al.* (12), 23 pretreatment tumor biopsies were evaluated by cDNA microarrays. The analysis revealed 54 to be genes differentially expressed between responders and non-responders, on the basis of downstaging ($p<0.001$). Using the leave-one-out cross-validation (LOOCV) method, 19 out of 23 patients had their response accurately predicted by their gene expression profiles ($p=0.02$). Using this method, 7 out of 9 responders and 12 out of 14 non-responders were correctly identified. In a validation set comprising 7 different tumor samples, 39 out of the original 54 genes identified from the training set were found to be differentially expressed. In the validation set, the gene expression profile was able to accurately predict response in six out of seven tumors.

Additionally, Rimkus *et al.* (13) evaluated pretreatment biopsies of 43 rectal cancer patients treated with neoadjuvant CRT. The microarray analysis revealed 42 genes to be differentially expressed among responders and non-responders, according to tumor regression grading. These 42

genes were identified from the 50 probe sets with the lowest *p*-values according to the Welch test. Using the LOOCV method, 10 out of 14 responders were correctly predicted, whereas 25 out of 29 non-responders were correctly predicted. In addition, 38 out of the 43 patients were selected randomly for a training set to develop a response classifier. This response classifier was used to predict response status in a small validation set consisting of five patients. The classifier predicted response in the validation-set patients with similar accuracy to the LOOCV method.

Both previous studies (12, 13) reported the ability to accurately determine responders and non-responders on the basis of microarray-determined gene expression profiles. However, between the 54 genes differentially expressed in the Ghadimi study and the 43 genes differentially expressed in the Rimkus study, there was no concordance, not even for a single gene. Furthermore, including our study, there was no concordance for any gene among the three studies. These studies, with a small number of patients, may not have sufficient power to validate the use of microarray-determined gene expression profiles to predict response to neoadjuvant CRT in rectal cancer. Kuremsky *et al.* (14), in a critical review of DNA microarray analysis, reported that although gene array expression data generate interesting results that may lead to the further exploration of candidate genes, the complexity and magnitude makes the results difficult to interpret.

Regarding the prediction of response to CRT using immunohistochemistry, Kuremsky *et al.* (14) reported that the six most commonly researched biomarkers evaluated were p53, epidermal growth factor receptor (EGFR), thymidylate synthase (TYMS), Ki-67, p21, and BCL-2/BAX. There is currently not enough evidence to suggest the clinical application of any biomarker to predict outcome in rectal cancer. We evaluated immunohistochemistry of *MMP7*, as a candidate gene, which showed the highest fold change in responders as compared with non-responders in histopathologic examination. Although MMP7 expression has not been previously described in rectal cancer as a biomarker to predict response of CRT, MMP7 appears to be a candidate biomarker, requiring future investigation.

The expression of *MMP7* in several types of cancer has been confirmed (15-17). The direct interaction of individual MMPs, particularly *MMP7*, with the genes and proteins involved in colorectal cancer development has been shown (18, 19). Specifically, the *MMP7* protein and its mRNA are also consistently expressed in liver metastases of colon cancer (18, 20). Our data suggest that preoperative CRT may be able to improve the prognosis of advanced rectal cancer patients with overexpression of *MMP7*.

Our validation of the gene expression patterns by microarrays revealed *TGFB1*, a gene related to apoptosis. *TGFB1* is a tumor suppressor gene. Barcellos-Hoff *et al.* showed in mice that activation of Tgf-b is an early and sensitive response to irradiation (21). In addition to acting as a tumor suppressor, TGFB has also been shown to have a pro-tumorigenic effect (22). Induction of Ras was shown to decrease the growth-inhibitory response to TGFB (23). There are reports showing that K-ras signaling may play a role in the conversion of TGFB from a tumor suppressor to a tumor promoter (24).

The present study defined the gene expression patterns for prediction of response to CRT with S-1 by customized and focused DNA microarray and validated its ability by immunohistochemistry and microarray using preoperative biopsy tissue samples in rectal cancer. Although the key weaknesses of this study are small sample, retrospective native and unsatisfactory analysis of validation, we evaluated gene expression patterns for prediction of response to CRT with S-1 by customized and focused DNA microarray. A multicenter randomized study for prediction of response to CRT (S-1 *vs.* UFT) by customized and focused DNA microarray is currently in progress. It will be necessary to confirm the usefulness of gene expression patterns for the prediction of response to CRT by larger prospective studies.

Conclusion

Gene expression patterns of diagnostic biopsies can predict pathological response to CRT with S-1 in rectal cancer.

Author Disclosures

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ORIGINAL

Intraperitoneal infusion of paclitaxel with S-1 for peritoneal metastasis of advanced gastric cancer : phase I study

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Abstract : Background : Intraperitoneal administration of taxanes revealed excellent anti-tumor effect for peritoneal metastasis of gastric cancer in some experimental models. The aim of this study is to determine maximum tolerated dose (MTD), dose limiting toxicity (DLT) and recommended dose (RD) of intraperitoneally infused paclitaxel (PTX) with S-1 as a phase I study. Patients and Methods : Eighteen patients with advanced gastric cancer in addition to confirmed peritoneal metastasis using laparoscopy were enrolled in this study. The regimen consists of oral administration of S-1 (Dose 80 mg : BSA< 1.25 m^2, 100 mg : 1.25<BSA<1.5 m^2, 120 mg : BSA>1.5 m^2) for 14 days and intraperitoneal infusion of PTX (Dose escalation : level I : 40, II : 60, III : 80, level IV : 90, V : 100 mg/m^2) at day1 and 14. PTX concentrations in serum and ascites were determined at 4, 8, 12, 24, 48 hours after the infusion, which was repeated twice every 4 weeks. Results : The number of patients were as follows : Level I : 3, Level II : 6, Level III : 3, Level IV : 3, Level V : 3. Grade 3 leukocytopenia was confirmed in 1 (Level II) and 2 (Level V). MTD is 90 mg/m^2, RD is 80 mg/m^2 and DLT is Grade 3 leukocytopenia. The average serum PTX concentrations remained in optimal range except for all 3 of level V patients. In all cohorts, the PTX concentrations in the ascites were approximately 1000 folds higher than those in serum for 48 hours after the infusion. Conclusions : MTD and RD were PTX 90 mg/m^2, 80 mg/m^2, respectively. These findings were supported by pharmocokinetics of PTX J. Med. Invest. 58 : 134-139, February, 2011

Mini-Abstract : *In intraperitoneal infusion of PTX with S-1, DLT was leukocytopenia, MTD and RD were PTX 90 mg/m^2, 80 mg/m^2, respectively. These findings were supported by pharmocokinetics of PTX*

Keywords : *paclitaxel, S-1, intraperitoneal infusion, peritoneal metastasis, gastric cancer*

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INTRODUCTION

Median survival time, even with the best supportive care, for patients with unresectable or metastatic gastric cancer is only 3.1 months (1). Although peritoneum is the most common metastatic site of

advanced gastric cancer, a standard regimen has not been established despite the number of trials and the survival rate is very low.

Recently new chemotherapy agents have been developed. In particular S-1 revealed a high response rate of 49% for advanced gastric cancer in late phase II study (2), which has been widely accepted as a key drug even for adjuvant setting in Japan (3).

Taxanes stabilize and excessively form microtubules, which is a different mechanism from other agents. In phase II study, response rate of paclitaxel (PTX) for advanced gastric cancer was 21% and not affected by differentiation of adenocarcinoma (4, 5). High concentrations approximately 1000 times of PTX in the peritoneal cavity maintained compared with those in serum after intraperitoneal administration because of fat solubility and heavy molecular weight ; 853.92 (6). Excellent pharmacokinetics and anti-tumor effect to the peritoneal dissemination of gastric cancer was reported in the experimental model (7).

It is considered that S-1 and PTX is one of the best combinations for the treatment peritoneal metastasis of gastric cancer. The aim of this study is to determine the appropriate doses and feasibility of intraperitoneal infusion of paclitaxel (PTX) with orally administered S-1.

PATIENTS AND METHODS

Patient eligibility

Patients with peritoneal metastasis of advanced gastric cancer were eligible for this clinical trial. Before initiation of the study, relevant study documentation was submitted to and approved by the responsible ethics committee : the University of Tokushima hospital clinical research Ethical Review Board, Tokushima, Japan.

The guidelines of the World Medical Association Declaration of Helsinki in its revised edition (Edinburgh, Scotland, October 2000) and other applicable regulatory requirements were strictly followed. Written informed consent was obtained from each patient before any study-specific screening procedures were undertaken.

Inclusion criteria

Patients aged 20-75 years, had to have histologically or cytologically confirmed peritoneal metastasis of gastric cancer using laparoscopy under general anesthesia, who had not received abdominal surgery and any prior chemotherapy regimens.

Exclusion criteria

Patients with ischemic heart disease that needed medication, liver cirrhosis, lung fibrosis, pneumonia, intestinal bleeding or other severe complications were excluded.

Treatment plan

An initial laparoscopy was performed under general anesthesia for the patients with advanced gastric cancer histologically diagnosed. Peritoneal metastasis was histologically confirmed by removal of disseminated nodules or peritoneal cytology.

The catheter for intraperitoneal infusion of PTX was passed through the wound of trocar port in the right side of the umbilicus, which was connected to the port implanted in the abdominal wall for the patient diagnosed peritoneal metastasis.

S-1 was orally administered with a fixed quantity (Dose 80 mg : Body Surface Area (BSA) < 1.25 m^2, 100 mg : 1.25 < BSA < 1.5 m^2, 120 mg : BSA > 1.5 m^2) for 14 days. PTX was infused intraperitoneally through the implanted catheter at day1 and 14. Dose of PTX was escalated ; level I : 40 mg/m^2, level II : 60 mg/m^2, level III : 80 mg/m^2 level IV : 90 mg/m^2, level V : 100 mg/m^2. Intraperitoneal PTX with S-1 was repeated two cycles every four weeks.

Adverse events were coded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. Dose Limiting Toxicity (DLT) was defined two patients had nonhematologic or hematologic grade 3 or greater adverse events. If one patient had Grade 3 or more adverse events, the cohort was expanded to three patients owing to occurrence of a DLT. As a result, the dose of PTX was increased to the level that two patients had a DLT in turn. The Maximum Tolerated Dose (MTD) was defined as one escalation level lower than that DLT was confirmed. Recommended dose (RD) was defined as one level lower than MTD.

Analytic methods and pharmocokinetics

Blood samples for pharmacokinetic analysis were drawn before infusion, at 4, 8, 12, 24 and 48 h after the infusion of PTX. Ascites samples were aspirated through the catheter for PTX infusion at the same time points. High performance liquid chromatography (Ultra-Violet absorbance detector : Ultra-violet of 227 nm in wave length) was used to analyze PTX concentrations of serum and ascites in SRL, Inc

(Tokyo, Japan).

RESULTS

Patient demographics

Patient demographics are shown in Table 1. The 18 patients were enrolled in this study after histologically confirming peritoneal metastasis. Two of the 18 patients had adenocarcninoma cells in peritoneal cytology without macroscopically detected metastatic nodules. Curative operation was not impossible for all 18 patients.

Table 1 : Patient demographics

Sex (male/female)	14/4
Age (years) (median/min/max)	56/49/75
WHO Performance status (0/1)	14/4
Macroscopic types III / IV	10/8
Histological typing well/poorly differentiated	3/15
Positive adenocarcinoma cells in peritoneal cytology	18
Macroscopically detected metastatic nodules	16
Gastrectomy	12

Clinical safety and tolerability

All 18 enrolled patients were evaluated for safety. A summary of the patient- and investigator-reported drug related clinical adverse events is shown in Table 2. Current regimen was generally well tolerated, with 6 patients clinically significant drug-related adverse events. The most frequently reported adverse event was Grade 3 leukocytopenia. Grade 1 or 2 anemia, vomiting and abdominal pain were confirmed.

The 40, 80, 90 and 100 mg/m^2 cohort enrolled three patients. After the one patient had Grade 3 leukocytopenia in 60 mg/m^2 cohort, this cohort was expanded to 6 patients without Grade 3 or more adverse events. Grade 3 leukocytopenia was confirmed consecutively 2 patients in 100 mg/m^2 cohort.

DLT was leukocytopenia, MTD was 90 mg/m^2 and RD was 80 mg/m^2, respectively.

Table 2 : Drug-related adverse events

	Grade 1	Grade 2	Grade 3	Grade 4
BLOOD/BONE MARROW				
Hemoglobin	1 (5.6%)			
Leukocytes			3 (16.7%)	
GASTROINTESTINAL				
Vomiting	1(5.6%)			
PAIN				
Abdominal pain		1 (5.6%)		

Pharmocokinetics of PTX

The average serum PTX concentrations in 40, 60, 80 and 90 mg/m^2 cohort were maintained between the lower limit of cytotoxic effects and upper limit of blood system disorder, which were over upper limit of blood system disorder in all 3 patients of 100 mg/m^2 cohort. In all cohorts, PTX concentrations in the ascites were approximately 1000 folds higher than those in serum for 48 hours after the infusion (Figure 1).

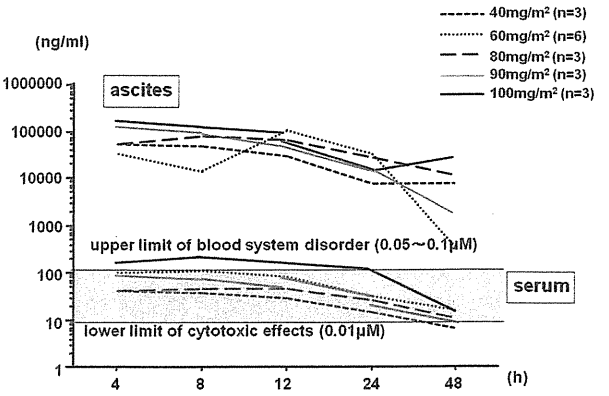

Figure 1 : Pharmacokinetics of PTX
The PTX concentrations in 40, 60, 80 and 90 mg/m^2 cohort remained in the optimal range. In 100 mg/m^2 cohort, the PTX concentrations were over upper limit of blood system. PTX concentrations in the ascites were approximately 1000 times higher than those in serum.

Clinical activity

All 18 patients were evaluated for efficacy. Objective clinical response was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST). The 2 patients had partial response. The 15 patients was recorded as stable disease, however, positive adenocarcinoma cells in peritoneal cytology became negative in 2 patients, remarkable decrease of ascites was found in 2 patients. Down staging according to the 13th Japanese Classification of Gastric Carcinoma was possible in 2 patients (2 : positive cytology became negative). There was one patient classified as having progressive disease.

Gastrectomy was performed for 12 of 18 patients, which had curative potential in the patients with down staging. The median survival time was 11 months. Survival time of the 2 patients whose positive cytology became negative was 32 and 48 months, respectively (Table 3).

Table 3 : Clinical activity

Case	Level	RECIST	Down staging	Gastrectomy	Prognosis	Survival time (months)
1	1	PR	-	+	death	8
2	1	SD	-	+	death	10
3	1	SD	+*	+	alive	48
4	2	SD	-	+	death	17
5	2	PR	-	+	death	21
6	2	SD	-	-	death	15
7	2	SD	-	-	death	14
8	2	SD	-	+	death	10
9	2	SD	-	-	death	11
10	3	SD	+*	+	alive	32
11	3	SD	-	+	alive	30
12	3	SD	-	+	death	8
13	4	SD	-	+	death	9
14	4	SD	-	-	death	6
15	4	SD	-	+	death	5
16	5	PD	-	-	death	14
17	5	SD	-	-	death	7
18	5	SD	-	+	alive	11

* Positive adenocarcinoma cells in peritoneal cytology became negative.

DISCUSSION

Intraperitoneal infusion of PTX was generally well tolerated. The most frequently reported adverse event was Grade 3 leukocytopenia. DLT was leukocytopenia, MTD was 90 mg/m^2 and RD was 80 mg/m^2, respectively. These findings were supported by pharmacokinetics of PTX.

Because S-1 is the most widely accepted drug for gastric cancer in Japan, a lot of combination trials based on S-1 have been performed (8-10). Median overall survival was significantly longer in patients assigned to S-1 plus cisplatin (13.0 months) than in those assigned to S-1 alone (11.0 months) in the Phase III trial for advanced gastric cancer, however, peritoneal dissemination held 34%, 24% of each group, respectively (8). Significant differences in overall survival compared with S-1 alone revealed in any other regimens. It has not been established standard regimens for peritoneal metastasis of gastric cancer.

Intraperitoneal PTX in the phase II trial for the patients with small-volume residual carcinomas of the ovary, fallopian tube, or peritoneum was well tolerated, which included only moderate abdominal pain (grade 2 : 15.7%, grade 3 ; 1.3%) and minimal neutropenia (grade 2 ; 3.9% ; grade 3 ; 1.3%) (11). The incidence of Grade 3 neutropenia were observed in 32% of the patients with advanced gastric cancer in the treatment schedule comprised an intravenous infusion of 80 mg/m^2 PTX , repeated weekly three times for 4 weeks (12). These data suggested that intraperitoneal administration of PTX did not increase the incidence of drug-induced toxicities (13).

A pharmacokinetics study demonstrated that the PTX concentration in ascites remained in the range of the lower limit of cytotoxic effects and upper limit of blood system disorder from 4 to 72 hours after intravenous infusion of 60 and 80 mg/m^2 PTX. On the other hand, plasma concentrations of PTX were over upper limit of blood system disorder at 4 hours (14). In contrast, the PTX concentrations in 40, 60, 80 and 90 mg/m^2 cohort in this study remained in the optimal range. In 100 mg/m^2 cohort, the PTX concentrations were over upper limit of blood system. PTX concentrations in the ascites were approximately 1,000 folds higher than those in serum.

A major advantage after intraperitoneal delivery of PTX is high concentration in the peritoneal cavity (550-2,000 folds) compared with the systemic compartment (13). Drug exposure of high concentration is considered to have an advantage because antitumor effects increased dose dependent manner

as far as could be seen there were no severe toxicities in the experimental model (7).

Although this study is a phase I study, the response rate and survival could not be described exactly, two patients with positive adnocarcinoma cells and no macroscopically detected disseminate nodules had a long survival of over 30 months. The overall 5-year survival (43.8%) of advanced gastric cancer patients with intraperitoneal free cancer cells without overt peritoneal metastasis (CY+/P-) after extensive intraoperative peritoneal lavage followed by the intraperitoneal chemotherapy (EIPL-IPC: peritoneal lavage of 10 times using 1 L of physiological saline following cisplatin at a dose of 100 mg/body into the peritoneal cavity) was significantly better than that of the intraperitoneal chemotherapy (4.6%) and the surgery alone (0%) (15). It is important to detect positive adenocarcinoma cells in the peritoneal cavity to improve survival of the patients with peritoneal metastasis (16).

Concerning patients with macroscopically detected peritoneal metastasis, the utility of peritonectomy with chemohyperthermic peritoneal perfusion (CHPP) was reported, however, there are some problems regarding peritonectomy : complicated procedures and CHPP : severe stress to the patients and needs of specific and expensive instruments (17).

Fat solubility of PTX is suitable for intraperitoneal infusion, in contrast, Cremophor EL and ethanol is necessary as a solvent for clinical use, which causes acute hypersensitivity (18). For better and safe drug delivery system, various modifications of PTX have been developed and phase I trials were reported (19-21). Intraperitoneal PTX using the water-soluble solvent revealed excellent pharmacokinetics compared with Cremophor EL (22).

Intraperitoneal PTX including new modified drugs has high potentials to improve survival for the peritoneal metastasis of gastric cancer.

CONFLICT OF INTEREST STATEMENT

Mitsuo Shimada received a research grant from Research Support Foundation of the University of Tokushima and TAIHO Pharmaceutical. Co. Ltd. ; Other authors have no conflict of interest.

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Histone deacetylase inhibitor enhances the anti-tumor effect of gemcitabine: A special reference to gene-expression microarray analysis

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Abstract. Histone deacetylase (HDAC) is strongly associated with epigenetic regulation and carcinogenesis, and its inhibitors induce the differentiation or apoptosis of cancer cells. Valproic acid (VPA) is one of the clinically available HDAC inhibitors. We investigated the anticancer effects of VPA in combination with gemcitabine (GEM) in the human cholangiocarcinoma cell line HuCCT1, and explored the mechanisms of the anticancer effects using microarray analysis. The anticancer effects of VPA or gemcitabine (GEM), and the effects of VPA combined with GEM, were studied by a cell proliferation assay. A microarray analysis was performed and the genes were picked up using GeneSpring GX11.5, followed by Ingenuity Pathways Analysis (IPA) and determination of gene expression by RT-PCR. GEM (5 nM) and VPA (0.5 mM) reduced proliferation by 23%, which significantly augmented the anticancer effect of GEM alone or VPA alone ($P<0.01$). Using microarray analysis, 43 genes were identified with the comparison between the GEM group and the GEM plus VPA combination group. Interactions were identified between genes of the 'Cellular Development' network relevant to the differentiation of cancer cells using IPA. Furthermore, GEM combined with VPA up-regulated the HLA-DRA expression compared to the single agents ($P<0.01$). VPA augmented the effects of GEM by enhancing the gene network mainly including HLA-DRA, possibly through the modification of cancer cell differentiation.

Introduction

Cholangiocarcinoma is a cancer arising from bile duct epithelium. This cancer is one of the most difficult diseases to treat and no standard chemotherapy has been established (1,2). Widely used chemotherapeutic regimens include gemcitabine (GEM), a nucleoside analogue of cytidine (2',2'-difluorodeoxycytidine; dFdC), which has a response rate of <30% (3). New agents and innovative therapeutic approaches are being investigated. Our group has researched the resistance of chemotherapy in biliary tract cancers (4).

Alterations in the epigenetic modulation of gene-expressions have been implicated in cancer development and progression. Histone acetylation is a post-translational modulation of the nucleosomal histones that affects chromatin structure and modulates gene-expression. Histone deacetylases (HDACs) comprise an ancient family of enzymes that play crucial roles in numerous biological processes (5) and expressions of HDACs are up-regulated in many tumor types (6,7). We reported that the survival rate for pancreatic cancer patients that were HDAC1-positive was significantly lower than for those that were HDAC1-negative and HDAC1 was considered to be a promising therapeutic target in pancreatic cancer (8). HDAC inhibitors induce the differentiation or apoptosis of cancer cells (9,10). Therefore, HDAC inhibitors are promising novel anticancer agents. In this study, we used valproic acid (VPA), which has been shown to have anticancer effects in various cancer models (11-13). However, no reports have focused on the alteration of gene-expression related to the anticancer effect of VPA in a bile duct cell line using microarray analysis.

The goal of this study was to investigate the anticancer effect of VPA in combination with GEM in a cholangiocarcinoma cell line and to identify the molecular mechanisms of the anticancer effect using microarray analysis.

Materials and methods

Cell lines and culture conditions. HuCCT1 (STR: http://www2.brc.riken.jp/lab/cell/str_start.shtml?cell_no=RCB1960) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. All cell lines were grown in RPMI-1640, supplemented with 10% fetal bovine serum (FBS), 70 μg/ml penicillin, 100 μg/ml streptomycin (complete medium) and maintained at 37˚C in a humidified

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Key words: cholangiocarcinoma, histone deacetylase inhibitor, valproic acid, microarray analysis, ingenuity pathways analysis

incubator with 5% CO_2 in the air. The cells were maintained for no longer than 12 weeks after recovery from frozen stock.

Reagents. Valproic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), kept at 4˚C and diluted in PBS as necessary at the time of use. Gemzar (gemcitabine hydrochloride) was purchased from Eli Lilly and Co. (Indianapolis, IN) and made fresh in 0.9% NaCl on the day of use.

Cell proliferation assay. All of the tumor cells ($5x10^3$) were seeded into 38-mm^2 wells of flat-bottomed 96-well plates in quadruplicate and allowed to adhere overnight. The spent medium was then removed and the cultures were re-fed with new medium (negative control) or medium, containing different concentrations of VPA and GEM. Incubation was continued for 72 h prior to adding the Cell Counting Kit-8. After 2 h, the optical density was measured at 450 nm with a microplate reader (Multiskan JX; Lab Systems).

Microarray analysis. Total RNA was isolated from both the stimulated and unstimulated cells (HuCCT1) using the RNeasy Mini kit (Qiagen, Valencia, CA). Relative purity was examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA expression was analyzed using the GeneChip® Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA), containing 28,869 oligonucleotide probes for known and unknown genes. First strand cDNA was synthesized from 300 ng of total RNA by using the GeneChip Whole Transcript (WT) cDNA Synthesis and Amplification kit (Affymetrix) according to the manufacturer's instructions. cRNA (10 μg) was input into the second-cycle cDNA reaction. cDNA was fragmented and end-labeled with the GeneChip WT Terminal Labeling kit (Affymetrix). Approximately 5.5 μg of fragmented and labeled DNA target was hybridized to the Affymetrix GeneChip Human Gene 1.0 ST Array at 45˚C for 17 h in a GeneChip Hybridization Oven 640 (Affymetrix) according to the manufacturer's recommendation. Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GeneChip Scanner 3000 7G (Affymetrix) and CEL files were then generated for each array. The microarray data were normalized by the GeneSpring GX 11.5 software (Agilent). The cut-off value was set at 0.5-2.0 for the ratio (>2.0, up-regulation; 0.5-2.0, no change; <0.5, down-regulation). The complete datasets were deposited in the Gene Expression Omnibus database (accession no. GSE27036).

Gene Ontology (GO) was analyzed using the GeneSpring GX 11.5 software (Agilent) and a P-value <0.05 was used for significance. The Ingenuity Pathway Analysis (IPA) 8.7 (http://www.ingenuity.com) was then used to determine the functional pathways associated with the set of differentially expressed genes between genotypes. IPA utilizes the knowledge in the literature about biological interactions among genes and proteins.

RNA isolation and quantitative real-time RT-PCR. The extracted RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time RT-PCR was performed using the Applied Biosystems 7500 Real-Time PCR System, TaqMan Gene Expression

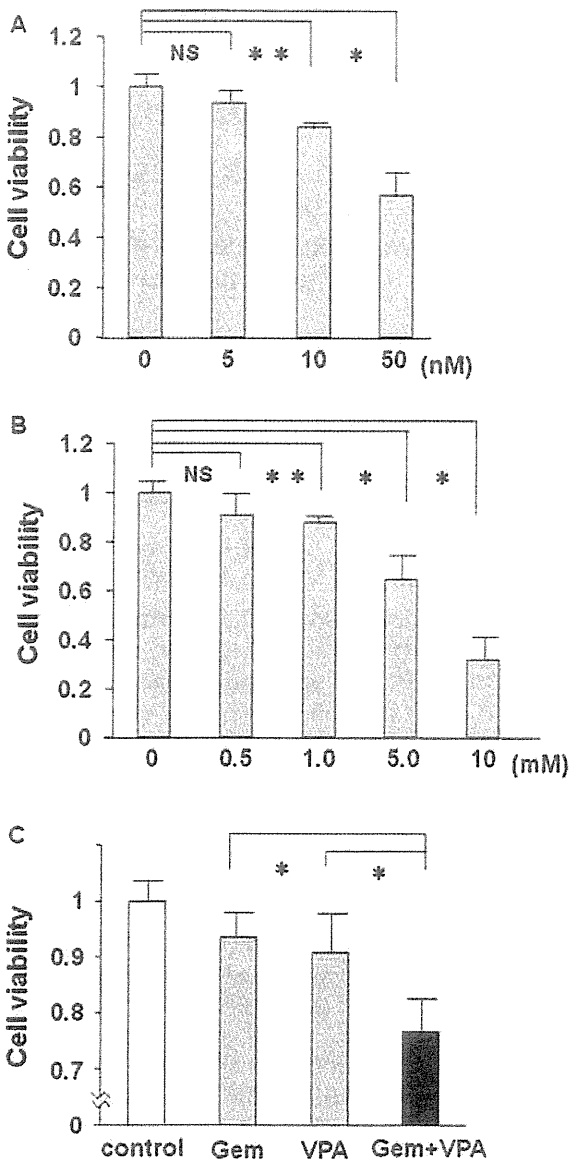

Figure 1. The effect of GEM (A), VPA (B) and the combination of GEM (5 nM) and VPA (0.5 mM) (C) on inhibiting cell proliferation of the human cholangiocarcinoma cell line, HuCCT1. *P<0.05; †P<0.01; NS, not significant.

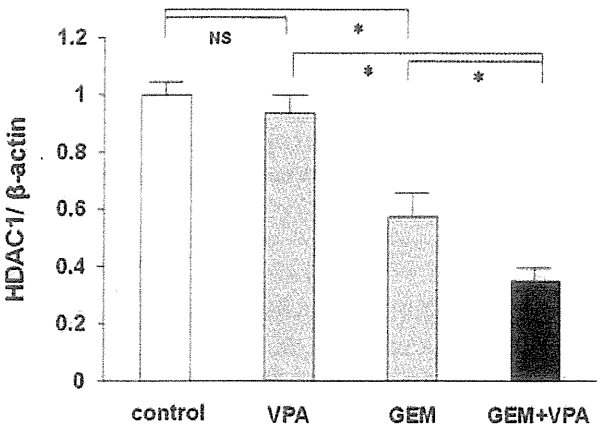

Figure 2. The gene-expressions of HDAC1 of human cholangiocarcinoma cell line, HuCCT1, in the combination of GEM (5 nM) and VPA (0.5 mM) †P<0.01.

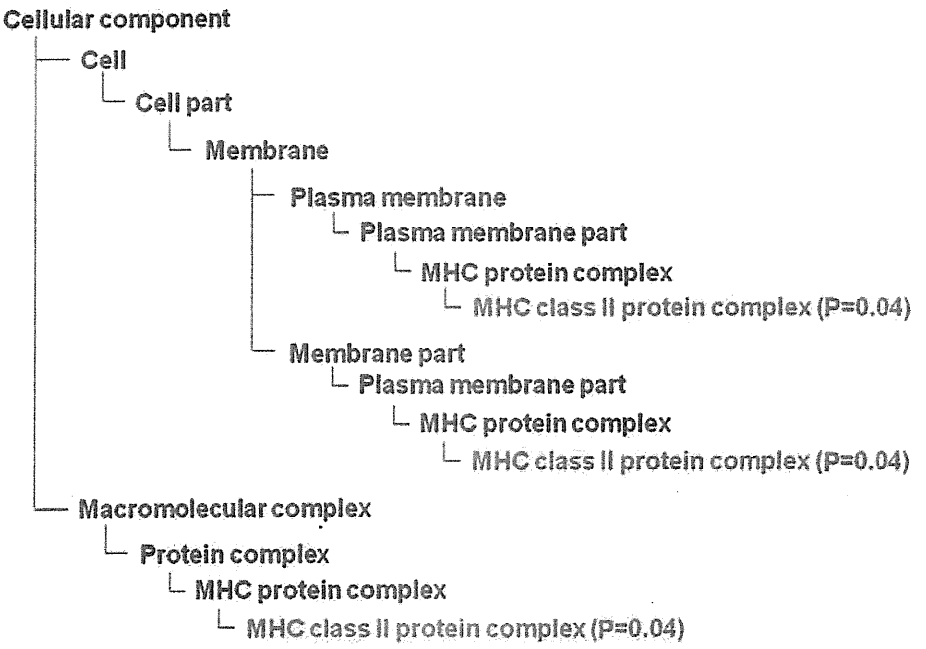

Figure 3. GO analysis with the comparison between GEM (5 nM) alone and GEM (5 nM) + VPA (0.5 mM) combination.

Table I. Differentially expressed genes by microarray analysis (GEM vs. GEM+VPA).

	Genes
Up-regulated (n=37)	ANKRD1, ANKRD22, CPM, LRRC49, CALB2, SERPINB7, CCDC88A, IQGAP2, CAMK4, ENPP1, PRKAR2B, SGCE, LRRCC1, HLA-DRA, HLA-DRB1, HLA-DRB3, HLA-DPB1, SELL, PLCE1, CNTN1, ARHGDIB, SILV, PHOSPHO1, PSG4, ATP6V1C2, MBOAT2, CALCRL, SPP1, SEPP1, SSBP2, CTGF, TSPAN13, PEG10, CD274, TSPAN7, FHL1, TMPRSS11E
Down-regulated (n=6)	HIST2H2BE, DPEP1, LGALS9, ABCA12, LCN2, NMI

Assays-on-demand and the TaqMan Universal Master mix (Applied Biosystems). The following assays were used: HDAC1 (no. Hs00606262_m1), HLA-DRA (no. Hs00219575_m1). The TaqMan Human ACTB endogenous control (4326315E) was used as a control gene. The thermal cycler conditions were as follows: 2 min at 50˚C, 10 min at 95˚C, 40 cycles of 15 sec at 95˚C, and 1 min at 60˚C. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection system version 1.3.1 (Applied Biosystems).

Statistical analyses. Statistical comparisons of mean values were conducted using one-way ANOVA. All the results are presented as mean ± SD. Statistical analysis was performed using StatView 5.0 J Software (SAS Institute, Inc., Cary, NC, USA). A P-value of <0.05 was considered to be statistically significant.

Results

In the GEM alone group, no effect of GEM was observed in the dose of 5 nM. Proliferation-inhibitory effects (16 and 43%) were observed at doses of 10 and 50 nM (Fig. 1A). In the VPA alone group, no effect of VPA was observed in the dose of 0.5 mM. Proliferation-inhibitory effects (12, 35 and 67%) were observed in doses of 1.0, 5.0 and 10 mM, respectively (Fig. 1B). GEM (5 nM) combined with VPA (0.5 mM) reduced proliferation by 23%, which significantly augmented the anti-cancer effects of GEM alone or VPA alone (P<0.01) (Fig. 1C). In regard to HDAC1 expression, GEM combined with VPA reduced the HDAC1 expression compared to GEM alone or VPA alone with real-time RT-PCR (Fig. 2).

Using microarray analysis, 683 and 357 unique genes were isolated in the GEM (5 nM) and VPA (0.5 mM) groups in comparison to the control group (fold change >2.0), respectively (data not shown). In addition, 43 differentially expressed genes were isolated between the GEM alone group and the GEM+VPA combination group (fold change >2.0) (Table I).

GO analysis revealed that the genes of the MHC class II protein complex showed significant different patterns (P=0.04) between the GEM alone group and the GEM+VPA combination group (Fig. 3). IPA-represented up-regulated (red) and down-regulated (green) genes in the gene network of the 'Cellular Development' contained the gene of the MHC class II protein complex, HLA-DRA, among the isolated genes between the two groups (Fig. 4).

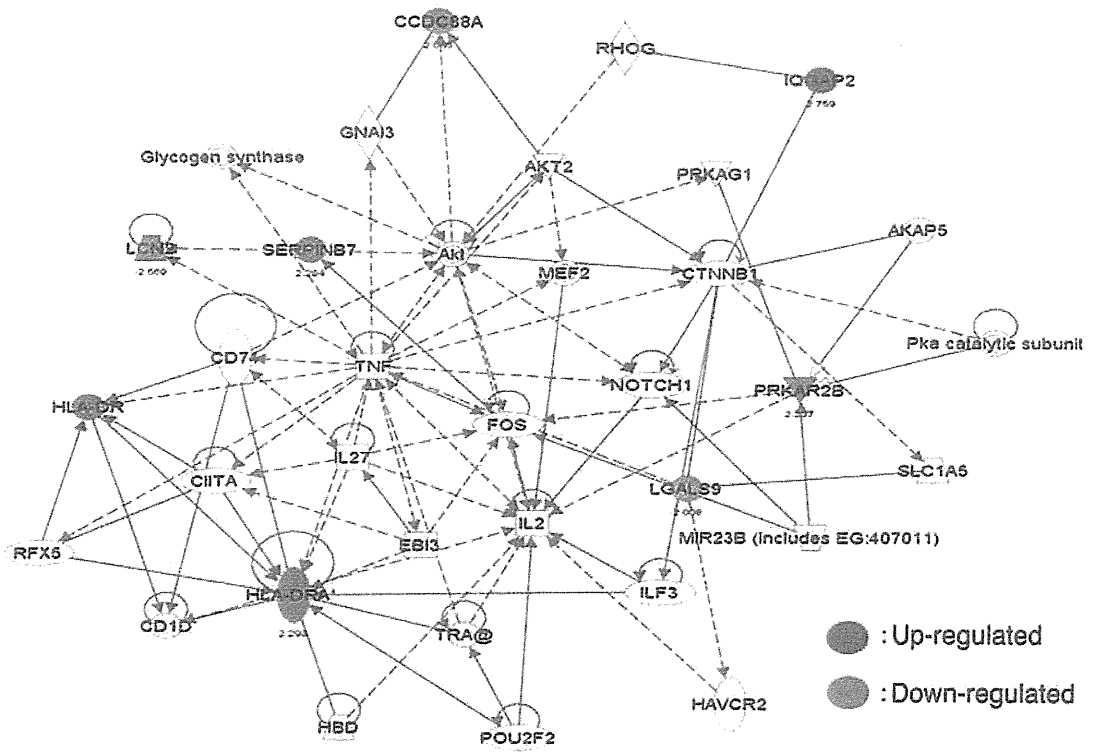

Figure 4. The gene network with the genes isolated from the comparison between GEM (5 nM) alone and GEM (5 nM) + VPA (0.5 mM) using IPA.

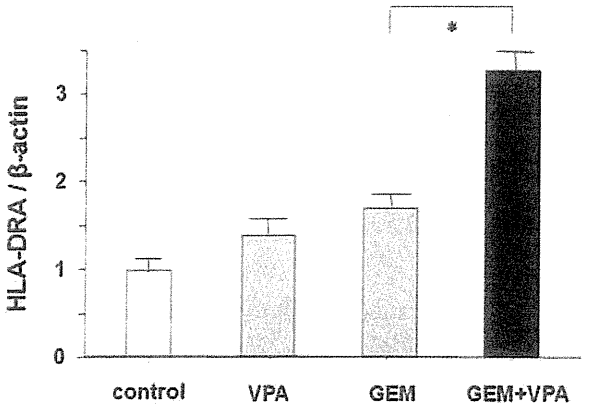

Figure 5. The gene-expressions in the HLA-DRA of human cholangiocarcinoma cell line, HuCCT1, with the combination of GEM (5 nM) and VPA (0.5 mM) *P<0.01.

Furthermore, up-regulation of the HLA-DRA expression in the group of GEM combined with VPA was confirmed by RT-PCR compared to the GEM alone or the VPA alone groups ($P<0.01$) (Fig. 5).

Discussion

In the present study, we assessed the effects of VPA, an HDAC inhibitor, in combination with GEM in the bile duct carcinoma cell line. To the best of our knowledge, this is the first report showing that VPA enhances the effects of GEM in a bile duct carcinoma cell line.

HDAC inhibitors have been shown to be useful in cancer therapy since they sensitize cancer cells to multiple chemotherapeutic agents (4,14). The HDAC inhibitor SAHA has been reported to enhance the effects of GEM in non-small cell lung cancer through the regulation of NF-κB (15). In the pancreatic cancer cell line, SAHA has been shown to augment the effects of GEM through up-regulation of p21 expression (16).

In this study, we utilized DNA microarray analysis and IPA to investigate the molecular mechanisms of the anticancer effects of VPA in combination with GEM. The microarray analysis may identify potential target genes for therapy, while IPA can be useful to understand the pathobiology at multiple levels by integrating data from a variety of experimental platforms and providing insight into the molecular and chemical interactions, cellular phenotypes and disease processes. We used IPA to analyze the gene network with the comparison between GEM only and the GEM+VPA combination. Using microarray analysis, VPA enhanced the anticancer effect of GEM through the gene network of the 'Cellular Development' containing HLA-DRA.

MHC class II molecules (HLA-DR, HLA-DP and HLA-DQ in humans) bind and display peptide antigens for recognition by $CD4^+$ T lymphocytes. MHC class II molecules play an important role in antitumor immunity (17,18). The expression of HLA-DR antigen in various cancers has been reported to influence carcinogenesis and prognosis (19-21) and the low expression of HLA-DR is an independent risk factor in HCC (22).

Furthermore, the expression of HLA-DR antigen in gastric cancer is associated with the degree of tumor cell differentiation (19,20). HDAC inhibitors can induce the differentiation of

solid tumors and reduce cell proliferation (23-25). Inhibition of the cell cycle is an essential event in cellular differentiation and the cytostatic effects of HDAC inhibitors are important for their anticancer activities. Apicidin, an HDAC inhibitor, caused the induction of stem cell differentiation into all three germ layers (26). In view of these findings, HDAC inhibitors have the potential to be used as differentiation-inducing therapy for cancer cells.

Many reports have documented the importance of histone acetylation as a positive regulator of MHC class II transcription (27,28) and HDAC inhibitors augmented the expression of the tumor cell MHC class II (29). On the other hand, HDAC activity repressed the MHC class II gene expression (30). In this study, after comparing the GEM alone group to the GEM and VPA combination group, the interactions were shown between genes of the 'Cellular Development' network containing the gene of the MHC class II protein, HLA-DRA. The HLA-DRA expression was up-regulated in the GEM plus VPA group. VPA may affect the differentiation of bile duct carcinoma cells because of the close correlation already shown between the expression of HLA-DR antigen and the differentiation of tumor cells (19,20). However, some of the HDAC inhibitors are of limited therapeutic use due to their toxic side effects at high doses (31). VPA is widely used as a therapeutic drug for epilepsy and its toxic profile and pharmacokinetic properties are well established. Furthermore, in our study, the promising dose of VPA was 0.5 mM, while the peak plasma concentration in patients treated for epilepsy ranging between 0.5 and 1.2 mM (32). VPA, at a dose of 0.5 mM, may not cause any serious side effects in clinical settings.

In conclusion, VPA augmented the therapeutic effects of GEM, at least partially by increasing the HLA-DRA expression. These effects may be associated with the modification of cancer cell differentiation.

Acknowledgements

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ORIGINAL

Effect of histone deacetylase inhibitor in combination with 5-fluorouracil on pancreas cancer and cholangiocarcinoma cell lines

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Abstract : Background : Histone deacetylase (HDAC) is well known to be associated with tumorigenesis through epigenetic regulation, and its inhibitors (HDACIs) induce differentiation and apoptosis of tumor cells. We examined the therapeutic effects of valproic acid (VPA, a HDACI) with a combination of 5-fluorouracil (5-FU) in vitro. Methods : A human pancreas cancer cell line (SUIT-2) and a cholangiocarcinoma cell line (HuCCT1) were used. Cell viabilities were evaluated by a cell proliferation assay. We determined the anticancer effects of VPA combined with 5-FU in these cell lines. Results : Pancreas cancer (SUIT-2) : No effect of 5-FU (1.0 μM) was observed, but 17% and 30% of proliferation-inhibitory effects were recognized in a dose of 2.5 or 5.0 μM, respectively. Cell viability was only weakly reduced by VPA (0.5 mM). However, in combination of 5-FU (1.0 μM) with VPA (0.5 mM), 19% of inhibitory effect was observed. Cholangiocarcinoma (HuCCT1) : 5-FU (1.0 μM) did not suppress the cell viability, but 5-FU (2.5 μM) suppressed by 23%. VPA (0.5 mM) did not suppress the cell viability, while VPA (1.0 mM) weakly decreased it by 11%. Combination of 5-FU (1.0 μM) and VPA (0.5 mM) markedly reduced the cell viability by 30%. Conclusion : VPA augmented the anti-tumor effects of 5-FU in cancer cell lines. Therefore, a combination therapy of 5-FU plus VPA may be a promising therapeutic option for patients with pancreas cancer and cholangiocarcinoma. J. Med. Invest. 58 : 106-109, February, 2011

Keywords *: pancreas cancer, cholangiocarcinoma, HDAC inhibitor, valproic acid, epigenetic regulation*

INTRODUCTION

Pancreas cancer is one of the most aggressive human cancers. The overall 5-year survival rate among patients with pancreatic cancer is < 5% (1). Cholangiocarcinoma is a cancer arising from bile duct epithelium. This cancer is one of the most difficult diseases to treat as pancreas cancer, and no standard chemotherapy has been established (2, 3). Therefore, we have researched about resistance of chemotherapy in pancreatic and biliary tract cancers.

5-fluorouracil (5-FU) is a chemotherapeutic drug which is widely used mainly for the treatment of the digestive system cancer, but the response rate

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in pancreatic and biliary tract cancers is very low (4, 5). Therefore, new agents and innovative approach to therapy are the important subjects for research.

Alterations in the epigenetic modulation of gene expression have been implicated in cancer development and progression, and histone acetylation, one of the epigenetic regulations, is a posttranslational modulation of the nucleosomal histones that affects chromatin structure and modulates gene expressions. Histone deacetylases (HDACs) comprise an ancient family of enzymes that play crucial roles in numerous biological processes (6), and HDACs are found to be overexpressed in many tumor types (7, 8). We reported that the survival rate for pancreas cancer patients with HDAC1-positive was significantly lower than that for patients with HDAC1-negative, and HDAC1 was considered to be a promising therapeutic target in pancreas cancer (9). HDAC inhibitors induce the differentiation or apoptosis of cancer cells (10, 11). Therefore, HDAC inhibitors are promising new agents, in this study, we used Valproic acid (VPA). VPA has the antitumor effects of a HDAC inhibitor (12), and VPA has been shown to have anticancer effects in various cancer models (13).

The aim of this study was to investigate the anticancer effects of VPA in combination with 5-FU in pancreas cancer and cholangiocarcinoma cell lines.

MATERIAL AND METHOD

Cell lines and culture conditions

SUIT-2 cell was purchased from the Japanese Collection Research Bioresources Cell Bank (Tokyo, Japan). HuCCT-1 was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 70 μg/mL penicillin and 100 μg/mL streptomycin (complete medium) and maintained at 37°C in a humidiWed incubator with 5% CO_2 in air. The cells were maintained for no longer than 12 weeks after recovery from frozen stock.

Reagents

Valproic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and kept at 4°C and diluted in PBS as necessary at the time of use. 5-FU was purchased from Kyowa Hakko (Tokyo, Japan) and made fresh in 0.9% NaCl on the day of use.

Cell proliferation assay

All of tumor cells (5×10^3) were seeded into 38-mm^2 wells of flat-bottomed 96-well plates in quadruplicate and allowed to adhere overnight. The spent medium was then removed, and the cultures were refed with new medium (negative control) or medium containing diVerent concentrations of VPA and 5-FU. Incubation was continued for 72 h prior to adding the Cell Counting Kit-8, and after 2 h, the optical density was measured at 450 nm with a microplate reader (Multiskan JX ; Labsystems).

Statistical analyses

Statistical comparisons of mean values were conducted using oneway ANOVA. All the results are presented as mean ± SD. Statistical analysis was performed using Stat View 5.0 J software (SAS Institute, Inc., Cary, NC, USA). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

In pancreas cancer cell line, SUIT-2, no effect of 5-FU was observed in dose of 1.0 μM and 17%, 30% and 33% of proliferation-inhibitory effects were observed in dose of 2.5, 5.0 and 10 μM (Fig. 1A). VPA (0.5 mM) weakly decreased cell viability by 13%, and VPA (1.0 mM) suppressed by 19% (Fig. 1B). In combination of 5-FU and VPA, 19% of inhibitory effect was observed in dose of 5-FU 1.0 μM/VPA 0.5 mM, the combination effect was significant compare

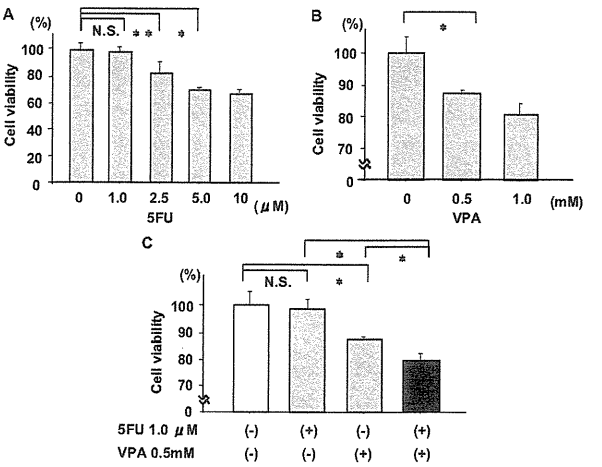

Figure 1 : The effect of 5-FU (A), VPA (B) and combination of 5-FU and VPA (C) in inhibiting cell proliferation of human pancreas cancer cell line, SUIT-2.
** : $p<0.05$, * : $p<0.01$.