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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

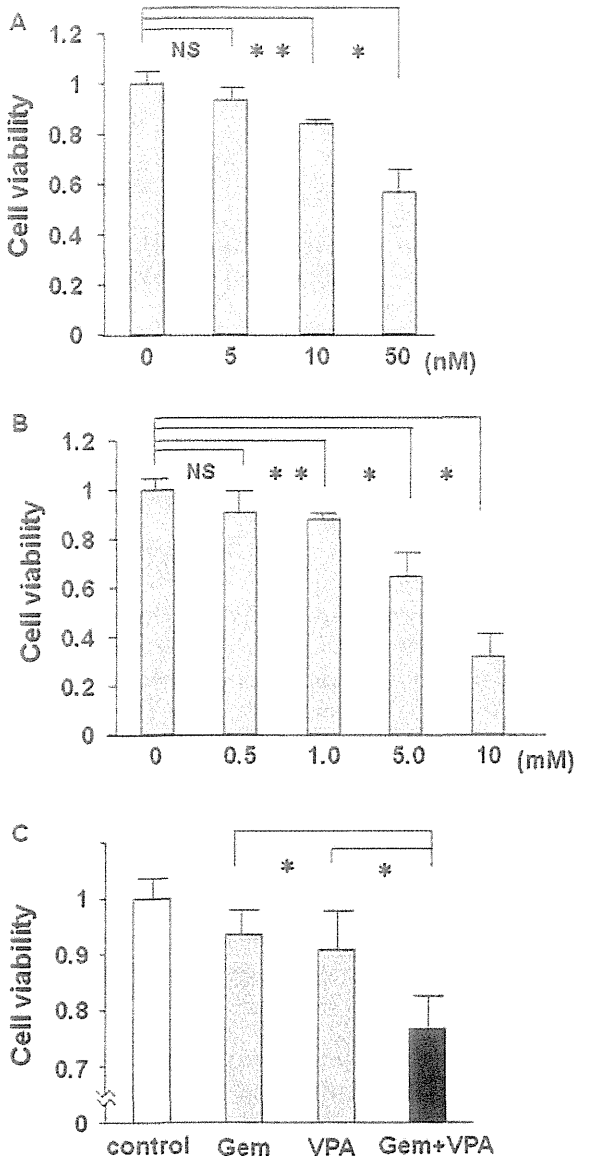

Figure 1. The effect of GEM (A), VPA (B) and the combination of GEM (5 nM) and VPA (0.5 mM) (C) on inhibiting cell proliferation of the human cholangiocarcinoma cell line, HuCCT1. *P<0.05; †P<0.01; NS, not significant.

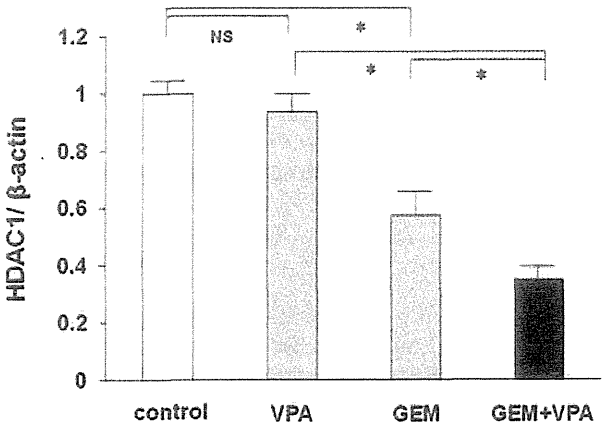

Figure 2. The gene-expressions of HDAC1 of human cholangiocarcinoma cell line, HuCCT1, in the combination of GEM (5 nM) and VPA (0.5 mM) †P<0.01.

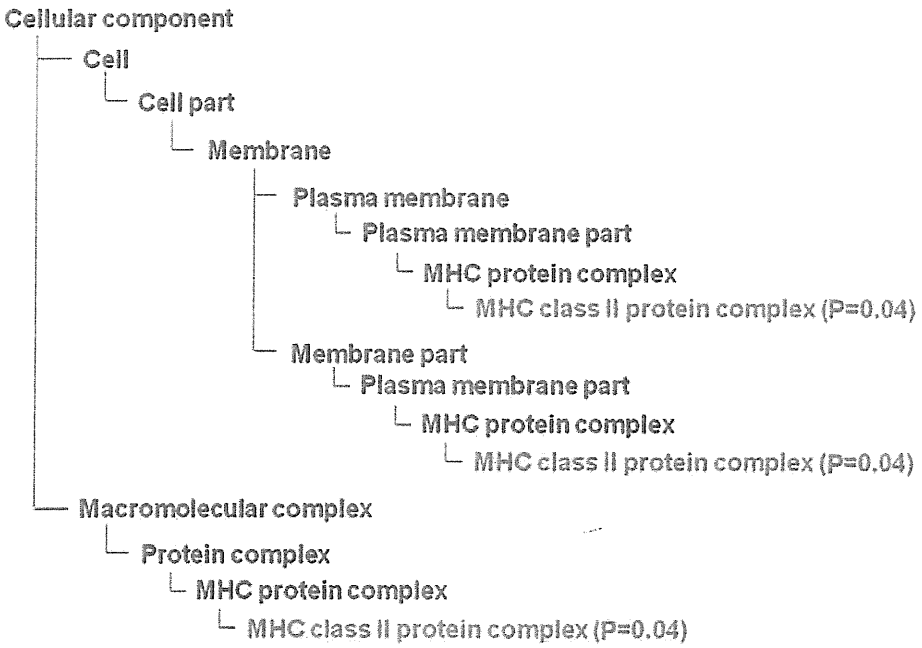

Figure 3. GO analysis with the comparison between GEM (5 nM) alone and GEM (5 nM) + VPA (0.5 mM) combination.

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).

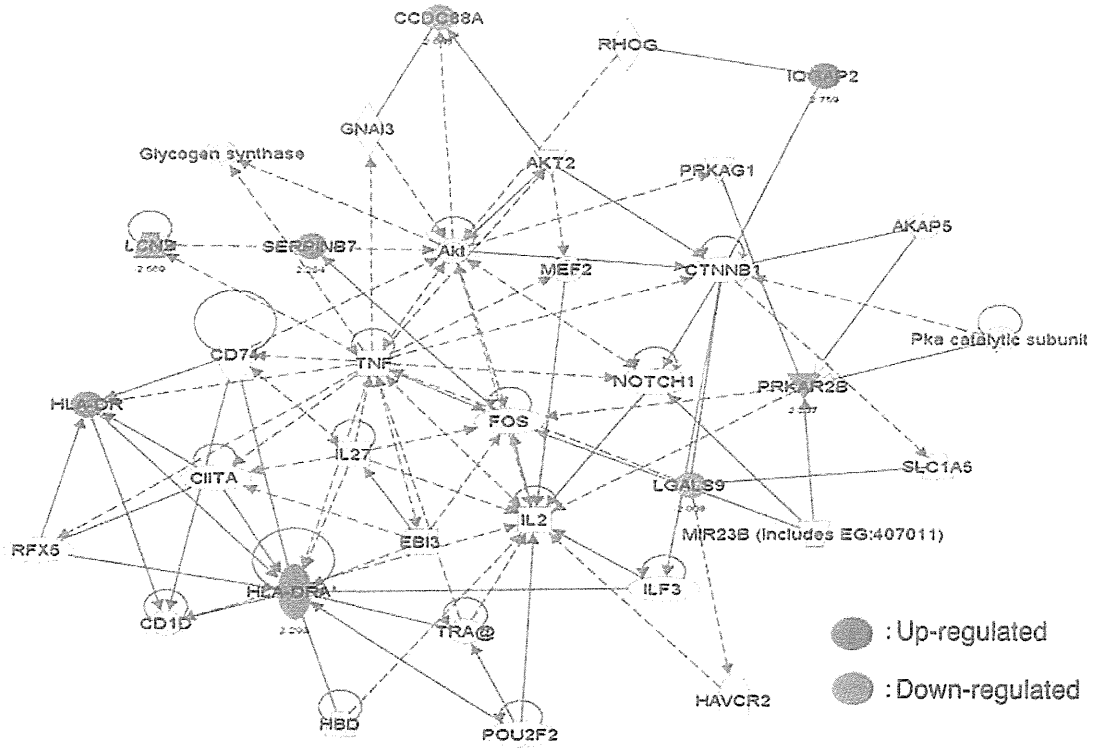

Figure 4. The gene network with the genes isolated from the comparison between GEM (5 nM) alone and GEM (5 nM) + VPA (0.5 mM) using IPA.

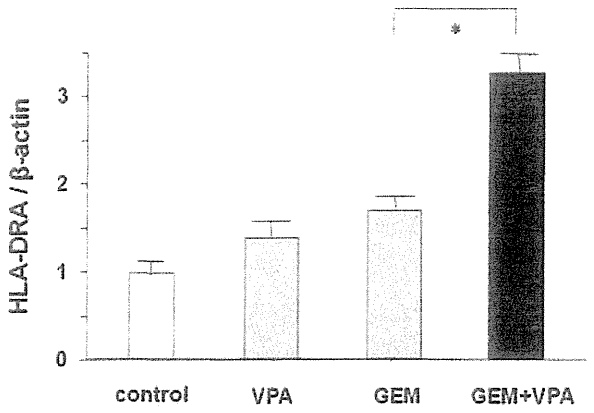

Figure 5. The gene-expressions in the HLA-DRA of human cholangiocarcinoma cell line, HuCCT1, with the combination of GEM (5 nM) and VPA (0.5 mM) *P<0.01.

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.

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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

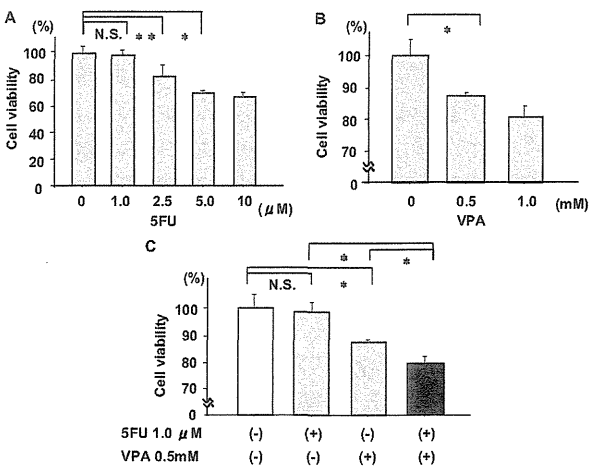

Figure 1 : The effect of 5-FU (A), VPA (B) and combination of 5-FU and VPA (C) in inhibiting cell proliferation of human pancreas cancer cell line, SUIT-2.
** : p< 0.05, * : p< 0.01.

to 5-FU alone or VPA alone ($P<0.01$) (Fig. 1C).

In cholangiocarcinoma cell line, 5-FU (1.0 μM) did not suppress the cell viability, 5-FU (2.5 μM) suppressed by 23%, and 34% and 39% of proliferation-inhibitory effects were observed in dose of 5.0 and 10 μM (Fig. 2A). VPA (0.5 mM) did not suppress the cell viability, while VPA (1.0 mM) weakly decreased it by 11% (Fig. 2B). 5-FU (1.0 μM) and VPA (0.5 mM) reduced by 30%, which significantly augmented the anticancer effect of 5-FU alone or VPA alone ($P<0.01$) (Fig. 2C).

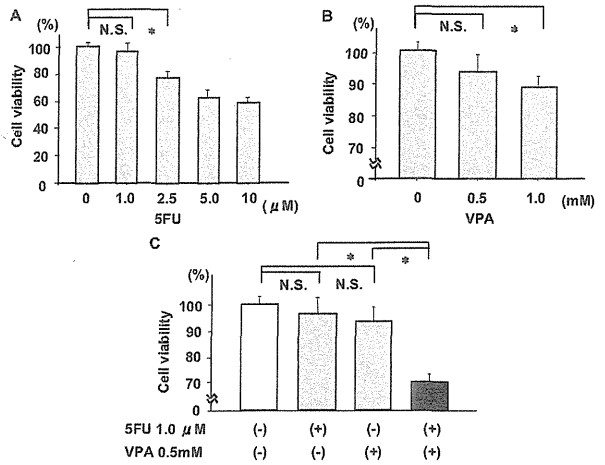

Figure 2 : The effect of 5-FU (A), VPA (B) and combination of 5-FU and VPA (C) in inhibiting cell proliferation of human cholangiocarcinoma cell line, HuCCT1.
* : $p<0.01$.

DISCUSSION

In the present study, we assessed the effect of HDAC inhibitor (VPA) in combination with 5-FU on pancreatic-biliary carcinoma cell lines. To our knowledge, this is the first report to show that VPA enhances the effect of 5-FU on both pancreas cancer and cholangiocarcinoma cell lines.

HDAC inhibitors are useful in cancer treatment when used in combination with current chemotherapeutic drugs, especially in combination with 5-FU, HDAC inhibitor (MS275) enhance the effect of 5-FU in colorectal cancer cells (14), and other HDAC inhibitor (SAHA) enhance the effect of 5-FU in non-small cell lung cancer (15). The mechanisms of the additional effects on HDAC inhibitors to the cytotoxic agent are the enhancement of apoptosis (14) and the up-regulation of p21(waf1/cip1) expression (15). In this study, the mechanisms may be the augmentation of apoptosis or the enhancement of p21 (waf1/cip1) expression.

However, some HDAC inhibitors are of limited therapeutic use due to toxic side effects at high doses (16). VPA is widely used as a therapeutic drug for epilepsy, its toxicity profile and pharmacokinetic properties are well established. Furthermore, in our study, the dose of VPA was 0.5 mM, because the peak plasma concentration in patients treated for epilepsy ranges between 0.5 and 1.2 mM (17). VPA at a dose of 0.5 mM may not cause any serious side effects in clinical setting.

Recently, S-1, an oral drug consisting of the 5-FU prodrug tegafur, combined with two modulators of 5-FU activity, has been developed (18-20). S-1 contains 5-chloro-2,4-dihydroxypyridine (CDHP), CDHP competitively inhibits the 5-FU degradative enzyme dihydropyrimidine dehydrogenase (DPD), resulting in the retention of a prolonged concentration of 5-FU in blood (18).

VPA has been investigated in clinical studies (21, 22), we plan the clinical trial of the combination therapy, S-1 and VPA. We have expected VPA enhances the anti-tumor effect of S-1 in this trial.

In conclusion, VPA augmented the inhibitory effects of 5-FU on the proliferation rates of both pancreas cancer and cholangiocarcinoma cell lines. Therefore, VPA in combination with 5-FU is suggested to be a promising therapeutic option for pancreatic and biliary tract cancers.

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特集I 胃癌化学療法

根治切除不能進行胃癌に対するTS-1＋Paclitaxel腹腔内投与*

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Key Words : paclitaxel, S-1, intraperitoneal infusion, peritoneal metastasis, gastric cancer

はじめに

進行胃癌腹膜播種症例は根治切除不可能であり，同様に予後不良とされる腹水洗浄細胞診陽性(以下CY1)症例とともに化学療法が選択されるが，多くの抗癌剤に対して抵抗を示し，必ずしも有効でない．これに対し直接的な効果を期待した投与法として抗癌剤の腹腔内投与の報告[1]がある．ただし，MitomycinC(MMC)やcisplatin(CDDP)などの水溶性薬剤は腹腔内投与後早期に腹膜や大網から吸収され，血中へ移行するため腹腔内濃度を長時間高濃度に維持できないこと，腹膜下組織の浸透距離の限界，腹腔内の均一な薬剤分布が期待できないといった理由から，期待した効果が得られないことがある．しかし，腹腔内投与に適した薬物動態を示す抗癌剤を選択すれば効果が期待でき，特にCY1症例の一部に対して有効であったという報告もある[2][3]．

近年CPT-11[4]，TS-1[5]，taxane系薬剤[6][7]といった胃癌に対し有効な薬剤が上梓され，5-FU単剤の有効性を超える薬剤として期待されている．そのなかでTS-1は本邦で新たに開発された経口5-FU系抗癌剤で，5FUのプロドラックであるテガフールにギメラシル[8](5-FU分解系酵素の可逆的拮抗阻害剤：抗腫瘍効果の増強)およびオテラシルカリウム[9](5-FU合成系酵素の可逆的阻害剤：消化器毒性の軽減)を配合することで，生体内の5-FU濃度を高めて抗腫瘍効果を増強し，また付随して増大する消化器毒性の軽減を達成した薬剤であり[10][11]，経口摂取可能な患者に対して大きな利便性を有することが特長である．TS-1単剤(4週間連日投与法)での胃癌に対する後期第II相臨床試験の奏効率は49％[12](25/51, MST：250日)，および44％[13](19/43, MST：207日)で，現在のところ単剤による治療成績としては最も高いものである．

また，paclitaxel(PTX)は，taxus brevifolia(イチイ科)の樹皮粗抽出物から単離された抗癌剤で，微小管蛋白重合を促進することにより微小管の安定化・過剰形成をひき起こし，その結果，細胞分裂を阻害し新規の抗腫瘍作用を有する薬剤として注目されるようになり，通常の投与法である経静脈的投与での胃癌に対する国内後期第II相試験[7]の結果では，奏効率は全体で23.3％と良好な成績が得られている．また，未分化型に対する奏効率は31％と報告され，胃癌腹膜播種症例に対するPTXの有用性が期待できると考えら

* Intraperitoneal infusion of paclitaxel with S-1 for case of non curative resection of advanced gastric cancer.

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れる．また分子量853.92を有し，脂溶性であることから腹腔内投与した場合のPTX腹腔内area under the curve(AUC)は血中AUCに比べ平均996倍(範囲336～2890倍)を示し，腹腔内の貯留時間が長い特性を示すことが明らか[14]となっており，腹腔内投与としても有効性を期待できる．PTXの腹腔内投与による報告としては卵巣癌を対象としたいくつかの報告[15-17]がある．そのなかでGynecologic Oncology Group(GOG)の行った第II相臨床試験の結果60mg/m²を毎週投与する方法において主な副作用としては腹痛(≧Grade 2)18%，末梢神経障害(≧Grade 2)8 %，顆粒球減少(≧Grade 2)6 %があり，経静脈的投与法に比べ安全性の高い投与法であることが確認されている．

しかし，PTXの腹腔内投与とTS-1併用療法の有効性や安全性は，報告が少ないため，今回われわれは腹膜播種あるいは他臓器浸潤胃癌症例を対象に，最大耐用量，用量制限毒性および推奨投与量について検討した．

対象・方法

研究者主導，施設単独の第Ⅰ相臨床試験を施行した．

対象被験者：2005年1月から2009年12月に経験した組織診または細胞診で胃癌の確定診断の得られた症例のうち，腹腔鏡検査で腹膜転移を認める(P1)，腹腔内洗浄細胞診で癌細胞を認める(CY1)または他臓器浸潤を認め，そのまま手術を行っても根治度Cとなると判断される胃癌症例20例を対象とした．症例の詳細選択基準は表1に示す．腹腔鏡検査の所見はP1：16例，CY1：2例，T4：2例であった．

TS-1は体表面積に応じて80mg/dayから120mg/dayを14日間内服し，14日間休薬した．PTXは40mg/m²から100mg/m²までdose escalationを行い，生理食塩液500mlに溶解し，腹腔内に投与した．投与後は15分間隔で体位を変え，2時間かけて腹腔内全体に薬液を接触させた．なお，腹水貯留例ではPTX投与前に可能な限り腹水のドレナージを行った．day 1, 14に腹腔内投与しday 8, 21は休薬した．4週間を1サイクルとして2サイクルのPTXとTS-1による化学療法を70日以内に終了した場合を，プロトコール治療完了と定義した．

表1　選択基準および除外診断

選択基準
①組織診または細胞診で胃癌の確定診断の得られた症例のうち，腹腔鏡検査で腹膜転移を認める(P1)，腹腔内洗浄細胞診で癌細胞を認める(CY1)または他臓器浸潤を認め，そのまま手術を行っても根治度Cと判断される胃癌症例．
②悪性腫瘍に対する前治療の既往がない症例．
③経口摂取が可能な症例．
④ECOGのperformance status(PS)が0-1の症例．
⑤年齢20歳以上75歳以下の症例．
⑥下記の主要臓器機能が十分保たれている症例．
⑦生存期間が3か月以上期待できる症例．
⑧重篤な合併症，活動性の重複癌のない症例．
⑨文書による同意の得られている症例．

除外診断：
重篤な合併症を有する症例．
治療を要する程度の虚血性心疾患，不整脈などの心疾患，心筋梗塞の既往，肝硬変，間質性肺炎，肺繊維症，消化管新鮮出血，精神障害，コントロール困難な糖尿病，腸閉塞，亜腸閉塞の症例．

たが，外来での治療を想定し2サイクルを通じてのMTD, DLTを検討した．MTD, DLTの評価は，PTX腹腔内投与後とTS-1経口投与終了後に行った(表2)．

検討項目は，primary endpoint(主要評価指標)として，用量制限毒性(dose limiting toxicity；DLT)，最大耐用量(maximaum tolerated dose；MTD)，推奨投与量(recommended dose；RD)とPTXの血中および腹水中の薬物動態を検討した．さらにsecondary endpoint(副次的評価指標)として抗腫瘍効果の確認(測定可能病変)，有害事象(副作用)を検討した．

PTXの血中および腹水中の薬物動態は1サイクル目のPTX投与後 4, 8, 12, 24, 48時間後に末梢静脈から5 ml採血し，腹腔内投与用ポートから腹水を5 ml採取し，それぞれ3000rpmで10分間遠心し，血清および腹水中PTX濃度を液々抽出HPLC法(SRL Tokyo, Japan)で測定した．

毒性評価は，NCI-CTCAE Ver 3.0日本語訳JCOG版を用いた．DLTおよびMTDの確認はPTX腹腔内投与後とTS-1経口投与終了後に施行した．なお，DLT, MTDの確認は2サイクルともに行った．Level 1より開始し，DLTが0例であれば次の投与量レベルへ移行した．1/3例の場合，同レ

表2-a　投与スケジュール

Paclitaxel＋TS-1投与スケジュール				
	1コース目			2コース目
	day 1	day14	day15～day28	day29
Paclitaxel 40～100mg/m²	↓	↓	休薬	↓
	day 1～14		day15～day28	day29～42
TS-1 80～120mg/body	↓ （2週間連日投与）		休薬 （2週間休薬）	↓ （2週間連日投与）

表2-b　TS-1およびTX投与のdose escalation

Dose escalation			
投与量Level	TS-1(mg/day)	PTX(mg/m²)	登録予定症例数
Level 1	80～120	40	3～6
Level 2	80～120	60	3～6
Level 3	80～120	80	3～6
Level 4	80～120	90	3～6
Level 5	80～120	100	3～6

ベルで3例追加し，6例合計で，1/6例であれば次のレベルへ移行した．2/6例以上であればMTDとした．2/3例では，MTDと判断し，MTDより1段階下げた用量をRDとした(表3)．

結　果

本試験では重篤な過敏症状は発現しなかった．

1．有害事象

有害事象はPTX40mg/m²で4例中Grade 1の嘔吐が1例，Hb減少を1例認め，60mg/m²で6例中Grade 2の腹痛が1例，白血球数減少を1例認めた．90mg/m²では4例中Grade 1の白血球数減少を1例認め，100mg/m²で3例中Grade 2の白血球数減少が1例，Grade 3の白血球数減少を2例認めた．以上の結果からDLTは白血球数減少，MTD：90mg/m²，RDは80mg/m²と考えられた(図1)．

2．血清中および腹水中PTX濃度の推移

PTXの薬物動態で血中濃度に関しては40mg/m²から90mg/m²は投与後4時間から48時間までの観察中は殺細胞効果発現濃度の10ng/m*l*から100ng/m*l*までを推移していたが，PTX100mg/m²は投与後4時間から24時間までは100ng/m*l*を越え，血液毒性発現濃度である0.05～0.1μMを推移し，24時間から48時間後には殺細胞効果発現濃度に低下した．この結果からMTDは90mg/m²と考えられた．

腹水中のPTX濃度はPTX40mg/m²から100mg/m²いずれの群も血清中の濃度の1,000倍程度のレベルで推移し，時間の経過とともに漸減するパターンを示した．いずれの群も有意差なく，ほぼ同様の腹水中の濃度を示した(図2)．

3．抗腫瘍効果

REIST分類ではpartial response(PR)が2例，stable disease(SD)が16例，progression disease(PD)が2例でresponse rateは10.0％であった．down stagingが可能であった症例はT4からT3になった症例(PR)が2例，CY陰転化した症例が2例で，down staging rateは20.0％であった．さらに腹水著効例は2例に認めた．化学療法後に胃切除術を施行したのは12例で，median survival rateは11か月でCYが陰転化した2症例の生存期間はそれぞれ32か月，48か月であった(表4)．

考　察

「胃癌治療ガイドライン第2版(2004年改訂)」では手術不能胃癌に対する化学療法の標準治療はなかったが，「胃癌治療ガイドライン第3版(2010年10月改訂)」では手術不能胃癌に対する化学療法としてTS-1とCDDPの併用療法が初回治療として推奨されるようになった．しかし，経口不可や中等量の腹水，腸管狭窄を呈している場合にはTS-1＋CDDPは必ずしも適応とならないことが多い．このような腹膜播種に対する化学療法は

表 3-a　用量制限毒性(DLT)の定義

用量制限毒性(DLT)	Grade 4 の白血球減少(1,000未満).
	Grade 4 の好中球減少(500未満). または38℃以上の発熱を伴うGrade 3 の好中球減少(1,000未満).
	Grade 3 以上の血小板減少(50,000未満).
	Grade 3 以上の非血液毒性(ただし, 脱毛は除く).
	3 週間以内にPaclitaxel投与が終了しなかった場合(1 回の投与延期までは可).

表 3-b　最大耐用量(MTD), 推奨投与量の決定

Dose escalation Levelの移行方法	
DLT発生状況	Levelの移行
当該投与量(Level)にてDLTが 0/3 例	次の投与量Levelへ移行する.
当該投与量(Level)にてDLTが 1/3 例	その投与量Levelでさらに 3 例を追加し, 追加症例を含めて 1/6 であれば次の投与量Levelへ移行する. それ以上であればそのLevelをMTDとする.
当該投与量(Level)にてDLTが 2/3 例以上	そのLevelをMTDとする.

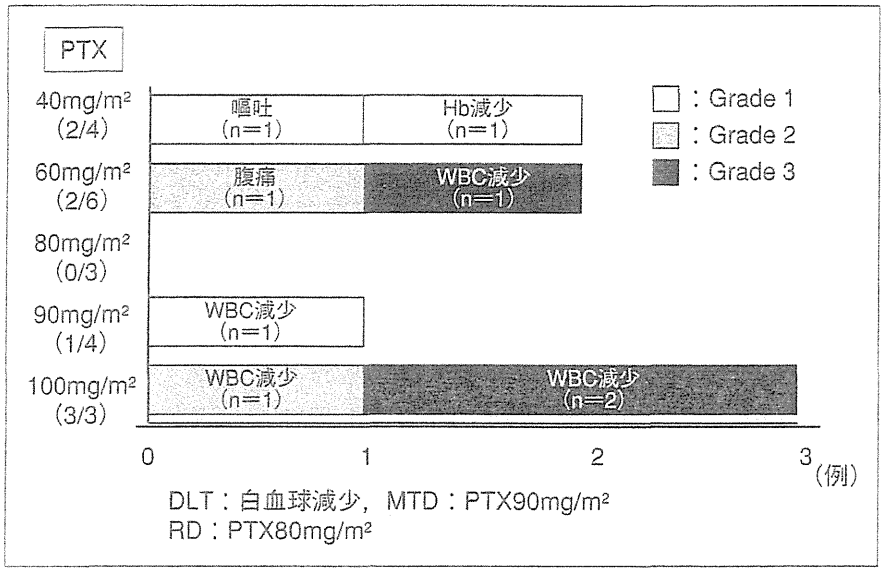

図 1　有害事象

有害事象についてgrade 3 の有害事象が 2 例発症したレベルがDLTレベルとした. Level 5 でgrade 3 の白血球減少が 2 例認められたため, DLTは白血球減少, MTDはPTX90mg/m²で, RD：PTX80mg/m²となった.

メトトレキサート(MTX)＋5FU, 5FU, taxane系薬剤など報告があるが, JCOG0106試験[18]の結果MTX＋5FUの5FU単独に対する優越性は検証できなかったことから, MTX＋5FUは積極的にガイドラインでは推奨されていない. 今回われわれが施行したPTX腹腔内投与法は, PTXが脂溶性で, 血漿AUC/腹腔内AUCの比率がPTXは0.00038, docetaxelは0.0071に対してCDDPは0.05～0.35であることから, PTXは腹腔内投与に適しているという報告[14]を応用したものである. しかし腹腔内投与されたPTXが腹膜表面から浸潤する距離は数百μmから数mmであるため, 大きい播種結節では薬剤が内部へ到達できない可能性がある[19]. これに対して波多江らはヤギへの腹腔内へのPTX投与後の胸管中のリンパ液の濃度は血漿中の濃度の約1,000倍であったと報告[20]していることからPTX腹腔内投与法は大網, 後腹膜に後発する腹膜播種には効率よくPTXを集積させる方法と考えら

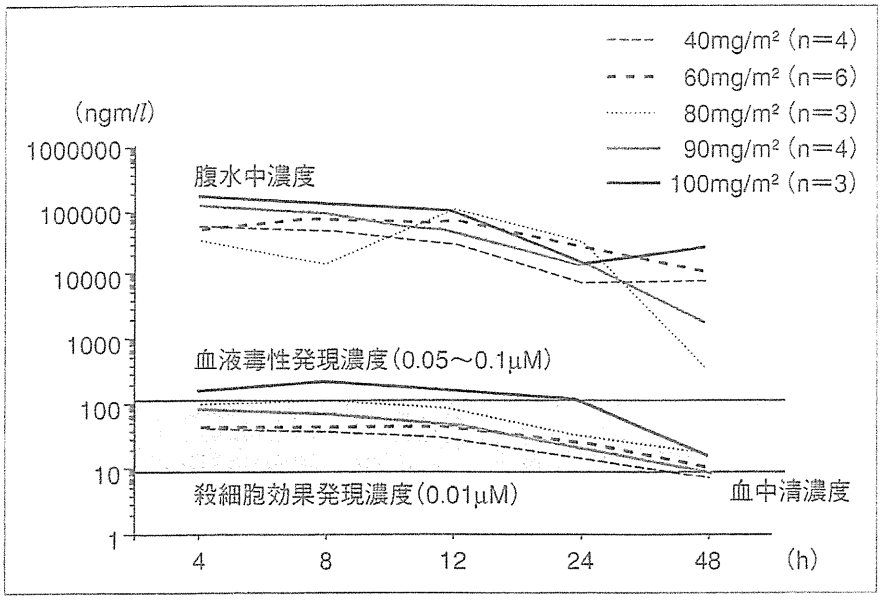

図2　Paclitaxelの薬物動態
薬物動態は，パクリタキセル100mg/m²を投与した症例で血液毒性発現濃度を超える血清濃度となった．腹水中濃度は血清中の1,000倍の濃度で推移していた．

表4　抗腫瘍効果

症例No.	Level	RECIST	Down staging	胃切除	予後	生存期間(月)
1	1	PR	−	+	死	8
2	1	SD	−	+	死	10
3	1	SD	+*	+	生	48
4	1	SD	+	+	生	18
5	2	SD	−	+	死	17
6	2	PR	−	+	死	21
7	2	SD	−	−	死	15
8	2	SD	−	−	死	14
9	2	SD	−	+	死	10
10	2	SD	−	−	死	11
11	3	SD	+*	+	生	32
12	3	SD	−	+	生	30
13	3	SD	−	+	死	8
14	4	PD	+	+	死	10
15	4	SD	−	+	死	9
16	4	SD	−	−	死	6
17	4	SD	−	+	死	5
18	5	PD	−	−	死	14
19	5	SD	−	−	死	7
20	5	SD	−	+	生	11

* 化学療法前腹水中の細胞診陽性症例．

れる．

近年，米国の大規模臨床試験でStage III卵巣癌術後の補助化学療法としてのPTX, CDDP腹腔内投与が全身投与に比較してMST(mean survival time)で16か月の増加をもたらすことが証明され，PTX腹腔内投与は進行卵巣癌に対する標準治療と認識されている[9][10]．本邦では東京大学で高度医療として2009年11月に承認を受けている．

一方，TS-1との併用療法に関しては，IshigamiらはTS-1：80mg/m²を2週間経口投与し，day 1とday 8にPTX20mg/m²を腹腔内投与と同日に50mg/m²の経静脈的投与を加えた第II相臨床試験

を40例に施行し，1年生存率が78%，2年生存率が47%，生存期間中央値が23か月で，治療前に癌性腹水を認めた21例中5例が腹水消失し，8例が減少し(有効率：62%)，奏効率は56%で副作用は白血球数減少：18%，悪心・嘔吐：10%と報告[2]しており，今回のわれわれの結果とほぼ同様であった．

本検討は第I相臨床試験ではあるため，response rateや生存率を論ずるには不十分ではあるが，CY1の2症例はいずれも30か月を越える長期間の生存を認めている．また，明らかな腹膜播種はないものの腹腔洗浄細胞診が陽性となった進行胃癌患者(CY+/P−)において，大量腹腔内洗浄および術中腹腔内抗癌剤投与(EIPL-IPC)の5年生存率の改善に対する効果の検討では，EIPL-IPC群の5年全生存率は43.8%であり，有意差をもって術中腹腔内抗癌剤投与(IPC)群(4.6%)やsurgery alone群(0%)より良好であった．さまざまな再発形式がみられるなかで，腹膜転移の発生率はEIPL-IPC群(40%)は他の2群(surgery alone群：89.7%，IPC群：79.3%)と比較して有意に低い結果が報告されている[3]．

以上より，根治切除不能進行胃癌に対するTS-1＋paclitaxel腹腔内投与併用療法は腹膜播種を予防する標準的治療として強く推奨される．

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医療費の削減と副作用の軽減を目指した modified OPTIMOX 療法

Modified OPTIMOX therapy aims at cut of medical expenses and reduction of side effects

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【ポイント】

◆ Modified OPTIMOX 療法は FOLFOX 療法と比較して無増悪生存期間および奏効率は同等であり，末梢神経障害を低率に抑える．

◆ ベバシズマブ併用にかかわらず modified OPTIMOX 療法と FOLFOX 療法では薬剤費に 170 万円の差を認める（治療期間 12 か月）．

◆ 薬剤費増加は保険財政を切迫し，患者負担も増加させるため，社会全体で考えていくべき問題である．

臨外　66(1)：40～44，2011

はじめに

大腸癌は，わが国における悪性腫瘍の罹患数の第 2 位（2003 年），死亡数の第 3 位（2007 年）であり，この 25 年間で罹患数は約 5 倍，死亡数は約 4 倍と，罹患・死亡ともに年々増加している[1]．このように増えている大腸癌であるが，昨今，実地臨床に，分子標的薬を含む多くの抗癌剤が導入されてくるようになった．それらの抗癌剤の薬価は一般的に高額であり，臨床上のベネフィットとリスクのみならず，経済学的な面からも十分に考慮して治療方針を決定する必要性が生まれている．

これまで抗癌薬の適応を決める場合，通常は生存期間の延長をプライマリ・エンドポイントとする臨床第Ⅲ相試験から得られたエビデンスをベースにしていた．しかし，本当に経済的な負担に見合った効果が期待できるのかという側面もきわめて大事である．われわれ医師は，使用しようとする抗癌剤の費用対効果はどのくらいであるのかを理解し，患者に対しても適切に情報開示をしたうえで，化学療法に関する同意を得る時代になってきているように思われる．

抗癌剤の費用対効果

抗癌剤によって得られる効果と経済的な負担のバランスを評価する指標の 1 つに「費用対効果」がある．標準的治療法と比較して分子標的薬併用で有意な生存期間の延長が得られる場合，全生存期間をアウトカム指標とした費用対効果を求めるには，その分子標的薬を併用することでどれだけの生存の延長が見込めるか〔増分効果（quality adjusted life year：QALY），life year gain（LYG）など〕と，その分子標的薬を用いることでどのくらい経済的負担が増加するか（増分費用）が必要となる．

計算式は，増分費用効果比（incremental cost effectiveness ratio：ICER＝増分効果/増分費用）で求められる[2]．費用効果分析における ICER の閾値はわが国では確固たる合意がないが，英国の National Institute for Health and Clinical Excellence（NICE）では約 480～720 万円/QALY を超えない治療が望ましいとされる．米国では約 510～1,020 万円/QALY という基準がしばしば用いられる．

STOP & GO strategy

■OPTIMOX1 療法

進行性大腸癌化学療法の key drug であるオキサリプラチン（L-OHP）はその蓄積性末梢神経障害が用量規定毒性であり，しばしば治療中止の要因となる．有効性を落とさずに末梢神経障害を減ずる方法として，すでに計画的な L-OHP 休止による STOP & GO strategy が報告されている（OPTIMOX1）．一方で，FOLFOX7 療法のうち L-OHP のみを休止する OPTIMOX1 を標準治療とし，化学療法を完全に休止する OPTIMOX2 に関しては，無増悪生存期間が劣ることが確認されており，持続静注 5-FU/LV 療法（sLV5FU2）による maintenance 療法の実施が適切であり，L-OHP 休止による STOP & GO strategy が末梢神経障害発現や重篤化の遅延をもたらし，L-OHP の有効性を最大限に用いる最善の方法であるといわれている．

しかしながら，OPTIMOX1 の問題点としては以下の 3 点が挙げられる．

①FOLFOX7 療法のオキサリプラチン量は mFOLFOX6 療法よりも多い．

②FOLFOX7 療法 6 クール，sLV5FU2 による maintenance 療法 12 クール，FOLFOX7 療法 6 クール施行して化学療法を終了している．

③sLV5FU2 による maintenance 療法が 12 クールと長期間である．

そこでわれわれは，医療費削減および副作用軽減を目指し，modified OPTIMOX 療法の有効性および安全性を検討するために，治癒切除不能な進行・再発大腸癌に対する FOLFOX 療法と OPTIMOX 療法の比較第Ⅱ相臨床試験を行うこととした．本稿では，その中間報告を行うとともに，医療費の観点からも検討する．

■Modified OPTIMOX 療法

Modified OPTIMOX 療法の原法と異なるポイントは，

①FOLFOX4，mFOLFOX6 のどちらでもよい

②OPTIMOX 群の FOLFOX はオキサリプラチンの量を増加させない

③オキサリプラチン抜きの期間を 6 サイクルに短くする

④progressive disease（PD）まで 6 サイクルごとに続けるようにする

の 4 点である（**図 1**）．

OPTIMOX 群の 27 例，FOLFOX 群の 28 例で行った

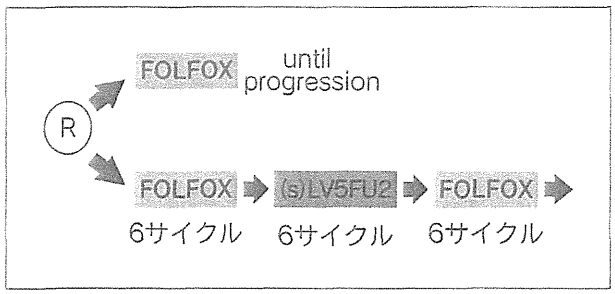

図 1　modified OPTIMOX 療法の試験デザイン
治癒切除不能な進行・再発大腸癌に対する FOLFOX 療法と OPTIMOX 療法の比較第Ⅱ相臨床試験（UMIN000002493）

表 1　化学療法の詳細

	OPTIMOX (n=27)	FOLFOX (n=28)	p value
FOLFOX レジメン			0.65
mFOLFOX6	23（85%）	25（89%）	
FOLFOX4	4（15%）	3（11%）	
Bevacizumab 併用			<0.01
あり	14（52%）	26（93%）	
なし	13（48%）	2（7%）	
コース数	12（1～32）	10（3～28）	0.72
累積 L-OHP 投与量 (mg/m2)	510 (85～1,530)	850 (255～2,380)	0.04

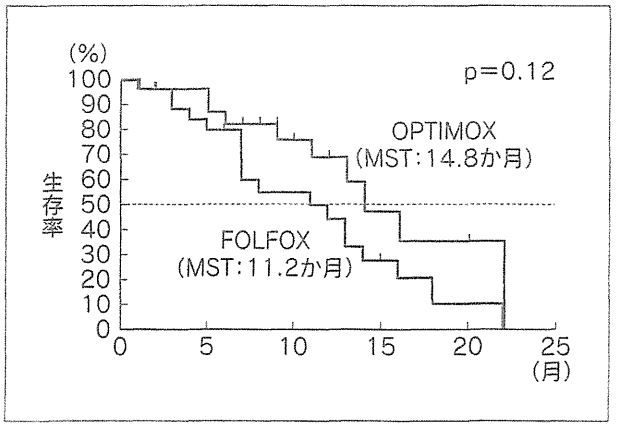

図 2　無増悪生存期間

臨床試験の結果は年齢，性別，performance status（PS），原発巣や転移巣部位に差は認めなかった．**表 1** に示すように，化学療法に関しては両群間で FOLFOX レジメンの違いとコース数には差は認めなかったが，ベバシズマブの併用率は FOLFOX 群で高率となっていた．また，オキサリプラチンの累積投与量は当然ながら OPTIMOX 群で少なくなっていた．

無増悪生存期間の median survival time（MST）は OPTIMOX 群で 14.8 か月，FOLFOX 群で 11.2 か月であり，差は認めなかった（**図 2**）．奏効率に関しても response rate，disease control rate は OPTIMOX 群で