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

Received for publication December 6, 2010 ; accepted December 28, 2010.

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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

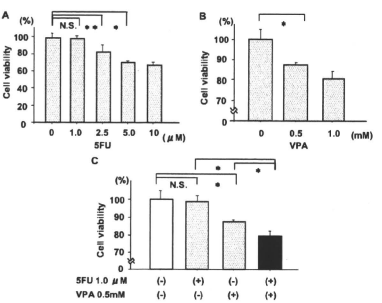

Figure 1 : The effect of 5-FU (A), VPA (B) and combination of 5-FU and VPA (C) in inhibiting cell proliferation of human pancreas cancer cell line, SUIT-2.
**: $p<0.05$, *: $p<0.01$.

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).

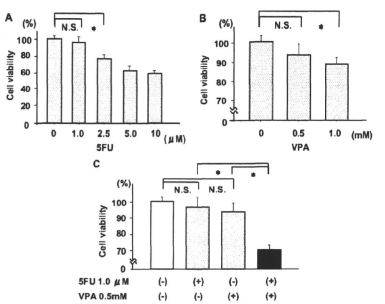

Figure 2 : The effect of 5-FU (A), VPA (B) and combination of 5-FU and VPA (C) in inhibiting cell proliferation of human cholangiocarcinoma cell line, HuCCT1.
* : $p<0.01$.

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.

ACKNOWLEDGEMENTS

Grant support was provided by the Grants-in-Aid for Scientific Researches of the Japan Society for the Promotion of Science (Grant-in-Aid for Young Scientists B : No. 22791286). We would like to thank Ms. Harada for providing technical assistance.

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Hepatology Research

Hepatology Research 2011; **41**: 64–70 doi: 10.1111/j.1872-034X.2010.00722.x

Original Article

Role of dihydropyrimidine dehydrogenase and thymidylate synthase expression in immunohistochemistry of intrahepatic cholangiocarcinoma

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Aims: Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) are key enzymes in the metabolism of 5-fluorouracil and have been implicated as possible prognostic markers for cancer patients. However, the clinical roles of DPD and TS in intrahepatic cholangiocarcinoma (IHCC) have not been investigated. The aim of this study was to clarify the clinicopathological role of DPD and TS expressions in IHCC.

Methods: Twenty-nine patients who had undergone hepatic resection for IHCC were enrolled in this study. Expressions of DPD and TS in the resected IHCC specimens were examined using anti-DPD or anti-TS antibody. The patients were divided into positive and negative groups according to DPD/TS expressions: DPD-positive group ($n = 18$) and DPD-negative group ($n = 11$)/TS-positive group ($n = 14$) and TS-negative group ($n = 15$). Clinicopathological factors were compared between the two groups.

Results: The overall survival rate was significantly lower in the DPD-negative group than in the DPD-positive group (1-year 36.4% vs. 77.4%, 3-year 18.2% vs. 43.0%; $P < 0.05$). The disease-free survival rate in the DPD-negative group tended to be lower than that in the DPD-positive group. The overall survival rate or disease-free survival rate did not appear to be associated with the TS-expression status. The Ki-67 labeling index in the DPD-negative group was significantly higher than that in the DPD-positive group ($16.9 \pm 3.2\%$ vs. $13.2 \pm 3.3\%$; $P < 0.05$).

Conclusions: The negative DPD expression was significantly associated with the enhanced tumor cell proliferation and poorer prognosis in patients with IHCC. DPD expression is a potential prognostic indicator for IHCC.

Key words: intrahepatic cholangiocarcinoma, Ki-67 index, prognosis, recurrent pattern

INTRODUCTION

INTRAHEPATIC CHOLANGIOCARCINOMA (IHCC) accounts for five percent of primary malignant liver tumors, arising from biliary epithelium,[1] and well known to be one of the most malignant solid tumors found in the digestive organs.[1–5] This highly malignant carcinoma is associated with lymph node metastasis, intrahepatic metastasis, peritoneal dissemination, bile duct invasion, and vascular invasion.[2–5] Prognosis of IHCC is very poor with a 5-year survival rate ranging from 25% to 35%.[1–5] Therefore, it is important to elucidate tumor characteristics and prognostic factors after surgical resection for IHCC.

Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) act as key enzymes of pyrimidine cascade and 5-fluorouracil (5-FU) metabolism.[6–8] In this cascade, 5-FU is first catabolised by DPD. TS decreases the synthesis of deoxythymine monophosphate from deoxyuridine monophosphate, and exhibits antitumor effects.[8]

Expressions of DPD and TS are correlated with the antitumor effects of 5-FU and 5-FU based chemotherapy, such as tegafur-uracil, and S-1.[9–11] Recently, DPD and TS have been reported to play an important role in various kinds of cancers. Down regulation of DPD gene expressions may enhance the negative prognostic effect in colorectal tumors[12] and ovarian cancer.[13]

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Received 6 June 2010; revision 16 July 2010; accepted 22 July 2010.

Overall survival was significantly better in the TS negative patients than in the TS positive patients among resected colon cancer patients.[14] High expression of TS in tumors enhanced distant metastasis after surgery.[15]

We have previously reported that a low expression of DPD mRNA was a poor prognostic factor in hepatocellular carcinoma (HCC).[16] However, to the best of our knowledge, only one *in vitro* study examined the DPD and TS expressions in IHCC cell lines.[17]

This is the first report evaluating the association of the DPD and TS expressions with the clinicopathological variables in surgical patients with IHCC.

PATIENTS AND METHODS

Patients

TWENTY-NINE PATIENTS who had undergone surgical resection for IHCC at Tokushima University Hospital between 1992 and 2009 were included in this study. There were 20 men and 9 women, with a mean age of 66.9 years (range, 43–84 years). In 19 patients (65.5%), hepatic lobectomy was performed. Lymph node dissections of the hepatoduodenal ligament and along the common hepatic artery or more extended lymphadenectomies were performed in 14 patients (48.3%). Extrahepatic bile duct resections were performed on 11 patients (37.9%). Consequently, 22 patients (75.9%) had received R0 or R1 resections. None of the patients received prior chemotherapy or irradiation before surgical resection. Mean follow-up period was 29 months (range, 2–111 months). The clinical stages were defined according to the Classification of Primary Liver Cancer Study Group of Japan.[18]

The current study was authorized in advance by the Institutional Review Board of the University of Tokushima, and all patients provided written informed consent.

Immunohistochemistry

The expressions of DPD and TS in the resected IHCC specimens were evaluated with using immunohistochemistry as described previously.[19,20] Surgical specimens were fixed in 10% formaldehyde embedded in paraffin and cut into 4-μm thick sections. Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Deparaffinized sections were retrieved by microwaving for 20 min. Endogenous peroxidase activity was blocked by soaking the sections in 0.3% hydrogen peroxide in methanol for 30 min. After washing with PBS, sections were placed in normal goat serum (2% in PBS) for 30 min to reduce nonspecific staining. The sections were subsequently incubated with DPD antibody (rabbit polyclonal, dilution 1:200; Taiho Pharmaceutical, Tokushima) or TS antibody (rabbit polyclonal, dilution 1:200; Taiho Pharmaceutical), overnight at 4 °C in moist chambers. The sections were incubated with goat anti-mouse immunoglobulin for 20 min and then with horseradish peroxidase-conjugated streptavidin complex (Histofine SAB-PO Kit; Biogenex Laboratories, Tokyo). To visualize immunoreactivity, diaminobenzidine/H_2O_2 (1 mg/mL) in PBS was used as the substrate. The sections were counter stained with hematoxyline, dehydrated with ethanol, and treated with xylene.

Assessment of DPD and TS staining was expressed as the percentage of stained cells in the cytoplasms out of total number of tumor cells and devided into two groups as follows: <5%; negative expression, ≥5%; positive expression (Fig. 1).[19] The assessment of immunohistochemistry was conducted without knowledge of the results of other experiments.

Determination of the Ki-67 labeling index

The correlation between the Ki-67 labeling index and DPD or TS expression was investigated. Determination of the Ki-67 labeling index was previously reported.[16] Five hundred tumor cells were counted in each 4-μm thick section. The Ki-67 labeling index was defined as the number of Ki-67 positive nuclei divided by total number of cancer cells, and expressed as a percentage.

Statistics

All statistical analysis was calculated through Stat View statistical software (Stat View 5.0; SAS Institute, Cary, NC). Relationships between DPD or TS expression and the clinicopathological variables were analyzed with the χ^2 test and Mann–Whitney *U*-test. Survival curves were calculated using the Kaplan–Meier method and compared using the Wilcoxon test. All significant factors by univariate analysis were included in the Cox's proportional hazards model of multivariate analysis to identify independent factors influencing survival. Statistical significance was defined as $P < 0.05$.

RESULTS

Immunohistochemistry

IN DPD EXPRESSION, there were 18 (62.1%) positive and 11 (37.9%) negative cases. Regarding TS expression, there were 14 (48.3%) positive and 15 (51.7%) negative cases.

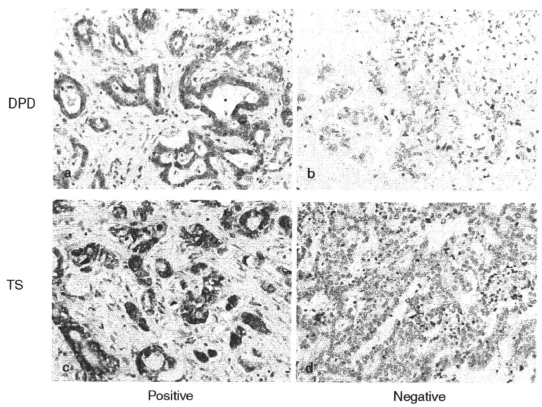

Figure 1 Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) expressions in intrahepatic cholangiocarcinoma (IHCC). The positive immunostaining of DPD or TS was recognized in cytoplasm of cancer cells (a) DPD-positive (b) DPD-negative (c) TS-positive (d) TS-negative.

Correlation between DPD/TS expressions and clinicopathological characteristics

No significant correlations were observed in any clinicopathological variables, such as staging, curability, vascular invasion, intrahepatic metastasis, and other tumor factors according to the expression levels of DPD or TS. However, in the DPD negative group, the tumor tended to be located more frequently in the hilar region (Table 1). In the TS positive group, the incidences of advanced clinical stage, non curative surgical resection, larger tumor size, vessels infiltration, and intrahepatic metastasis tended to be higher than in the TS negative group (Table 2).

Overall and disease-free survival according to DPD/TS expressions

Figure 2 shows overall and disease-free survival rates according to DPD/TS expressions. The overall survival rate was significantly lower in the DPD-negative group than in the DPD positive group (1-year 36.4% vs. 77.4%, 3-year 18.2% vs. 43.0%; $P < 0.05$) (Fig. 2A). However, there were no differences in the overall survival rate between the TS-negative and the TS-positive group (1-year 58.2% vs. 64.3%, 3-year 39.9% vs. 26.8%; Fig. 2B).

Similarly, the disease-free survival rate in the DPD-negative group tended to be lower than in the DPD-positive group (1-year 22.2% vs. 57.1%, 3-year 11.1% vs. 31.2%), although there was no statistical significance

Table 1 Clinicopathological characteristics according to dihydropyrimidine dehydrogenase (DPD) expression

Factors	DPD expression		*P*-value
	Positive ($n = 18$)	Negative ($n = 11$)	
Mean age (years)	68.3 ± 7.5	64.5 ± 13.7	0.448
Sex (Male/Female)	13/5	7/4	0.628
Virus ([-]/HBV/HCV/Combined)	9/4/4/1	10/1/0/0	0.140
Staging (I, II/III, IV)	5/13	3/8	0.976
Curability (R0, 1/2)	14/4	8/3	0.758
Location (hilar/peripheral)	4/14	5/6	0.190
Tumor diameter (<4 cm/≥4 cm)	9/9	5/6	0.812
Macroscopic type: T/T + I	9/9	4/7	0.474
Differentiation: Diff./Undiff.	7/11	4/7	0.892
LN metastasis: −/+	6/12	4/7	0.868
Vessels infiltration: −/+	7/11	6/5	0.412
Intrahepatic metastasis: −/+	12/6	9/2	0.376

Diff, differentiation; T, mass-forming type; T + I, mass-forming + periductal infiltrative type; Undiff, undifferentiation.

Table 2 Clinicopathological characteristics according to TS expression

Factors	TS expression		*P*-value
	Positive ($n = 14$)	Negative ($n = 15$)	
Age: Mean	68.3 ± 7.5	64.5 ± 13.7	0.448
Gender: Male/Female	10/4	10/5	0.782
Virus:(−)/HBV/HCV/ Combined	9/2/3/0	10/3/1/1	0.528
Staging: I, II/III, IV	2/12	6/9	0.122
Curability: R0, 1/2	9/5	13/2	0.159
Location: Hilar/ Peripheral	4/10	5/10	0.782
Tumor diameter: <4 cm/ ≥4 cm	5/9	9/6	0.191
Macroscopic type: T/T + I	5/9	8/7	0.340
Differentiation: Diff./ Undiff.	4/10	7/8	0.316
LN metastasis: −/+	9/5	10/5	0.893
Vessels infiltration: −/+	4/10	9/6	0.089
Intrahepatic metastasis: −/+	8/6	13/2	0.076

Diff, differentiation; T, mass-forming type; T + I, mass-forming + periductal infiltrative type; Undiff, undifferentiation.

(Fig. 2C). No significant difference in the disease-free survival rate was observed between the TS-negative and the TS-positive group (1-year 46.2% vs. 40.0%, 3-year 23.1% vs. 20.0%; Fig. 2D).

Univariate and multivariate analysis of prognostic factors

Table 3 shows the results of univariate and multivariate analysis of prognostic factors. Univariate analysis revealed that location, differentiation, macroscopic type, vessels infiltration and intrahepatic metastasis were not significant factors in terms of postoperative survival. In contrast, curability ($P = 0.0009$), tumor size ($P = 0.0066$), lymph nodes metastases ($P = 0.0127$), and negative expression of DPD ($P = 0.0498$) were found to be significant prognostic factors for survival after surgical resection. In multivariate analysis using the Cox's proportional hazard model, tumor size (≥4 cm) was found to be an only independent prognostic factor. Negative expression of DPD tended to be an independent prognostic factor, although there was no statistical significance ($P = 0.1293$).

Recurrent pattern

Table 4 shows the correlation between the recurrent patterns and the DPD expression status. The recurrence rate in the DPD-positive group was similar to that in the DPD-negative group. However, in the DPD-negative group, the incidence of recurrence in the liver was significantly higher ($P < 0.05$) and that of lymph node and remote organ tended to be higher compared to the DPD-positive group.

Ki-67 proliferating index

The Ki-67 labeling index in the DPD-negative group was significantly higher than the DPD-positive group (16.9 ± 3.2% vs.13.2 ± 3.3%) ($P < 0.05$). There was no difference in the Ki-67 labeling index between the TS-positive group and the TS-negative group (14.1 ± 3.8% vs.15.5 ± 3.5%).

DISCUSSION

THESE RESULTS SHOW that negative DPD expression was significantly associated with poorer prognosis, higher proliferation index, and a higher incidence of recurrence in the liver. However, TS expression was not related to patient prognosis after surgical resection.

Several other reports have documented that the expression of DPD is related to prognostic and clinicopathological factors.[12,13] In primary liver cancer, however, only a few reports are available on the role of DPD.[21,22] The DPD activity in HCC was lower than in non cancerous tissue and a gradual decrease in DPD activity was associated with liver damage.[21] We previously reported that low mRNA expression of DPD was a poor prognostic factor and significantly related to advanced clinical stage, undifferentiated histology, microscopic intrahepatic metastasis, and related to tumor proliferation in HCC.[16] Regarding biliary tract cancer, DPD concentration was higher in cancerous tissue than in noncancerous tissue, although the prognosis is not different.[23] Ajiki *et al.* reported DPD expression was not a prognostic factor in gallbladder cancer.[24]

In this study, the patients in DPD-negative group had a significantly poorer prognosis. The mechanism of such results is not fully understood at present. However, in pyrimidine cascade, negative expression of DPD leads the synthesis of both uracil and thymine. Increased synthesis of uracil and thymine may relate to the enhancement of pyrimidine nucleotide pools[25] and may cause the cell proliferation of IHCC. Johnston SJ *et al.* suggested that the down-regulation of DPD expression might create a favorable environment for tumor growth.[26] Further, it was suggested that low DPD expression was associated with an increase in the metastatic activity. DPD activity in highly malignant murine neu-

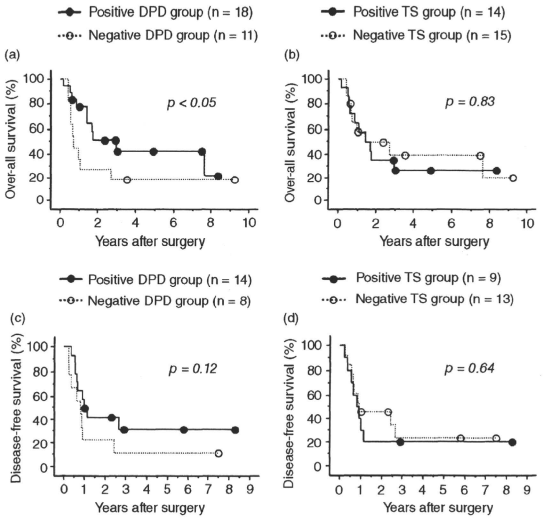

Figure 2 Overall survival rates in positive and negative expression of dihydropyrimidine dehydrogenase (DPD; a) and thymidylate synthase (TS; b). The survival rate in DPD-negative group was significantly lower than in the DPD-positive group. Disease-free survival rates in positive and negative expression of (c) DPD and (d) TS.

roblastoma cell line was lower than that in low malignant cell line.[27,28] Consistent with these reports, DPD-negative group had a significantly higher Ki-67 labeling index and higher incidence of recurrence in the liver than the DPD-positive group in our study.

Table 3 Univariate and Multivariate analysis of prognostic factors

	DP D positive ($n = 14$)	DPD negative ($n = 8$)	*P*-value
Recurrence	9	6	0.604
Recurrent pattern			
Liver ($n = 12$)	9	3	0.018
Lymph nodes ($n = 4$)	1	3	0.095
Peritoneum ($n = 1$)	0	1	0.205
Remote organ ($n = 2$)	0	2	0.063

Table 4 Correlation between DPD expression and recurrent site in patients with R0/1 resection ($n = 22$)

	Univariate *P*-value	Hazard ratio	Multivariate 95% C.I.	*P*-value
Curability: R2	0.0009	2.832	0.804–10.000	0.1052
Tumor Diameter: ≥4 cm	0.0066	4.413	1.427–7.705	0.0099
Lymph nodes metastasis: +	0.0127	2.921	0.851–10.030	0.0885
DPD expression: –	0.0498	2.132	0.801–5.672	0.1293

†F + I, mass-forming + periductal infiltrative type.

TS also plays an important role in pyrimidine cascade, and is implicated to be associated with tumor characteristics in many cancers.[14,15,29,30] TS gene expressions in primary gastric cancer differ according to degree of tumor cell differentiation.[29] High-TS expression was associated with poor survival in patients with colon cancer, and increased TS activity was related to proliferation of tumor cells.[30] In primary liver cancer, Baba *et al.* reported TS activity was generally lower in HCC.[21] However, TS expression was not related to the prognosis of patients with HCC in our previous report.[16] In biliary tract cancer, TS concentration was higher in cancerous tissue than in noncancerous tissue, and the patients with high-TS concentration had a better disease-free survival rate.[23] However, there is a report suggesting that TS may not be a prognostic factor in gallbladder cancer.[24] In pyrimidine cascade, TS does not affect synthesis of deoxythymine monophosphate via thimidine, or metabolism of deoxythymine monophosphate.[8] Therefore, it seems that TS is insufficient to alter the DNA synthesis and tumor cell proliferation in IHCC.

In conclusion, the negative DPD expression was significantly associated with the increased tumor cell proliferation and poorer prognosis in IHCC patients. We expect that DPD expression may be a useful biomarker in the prediction IHCC patient prognosis.

ACKNOWLEDGMENTS

THIS WORK WAS supported in part by the following grants: a Cancer Research Project by TAIHO Pharmaceutical and The University of Tokushima, a Grant-in-Aid for challenging Exploratory Research (No. 22659233), and a Grant-in-Aid for Young Scientists (B) (No. 21791292) from the Japan Society for the Promotion of Science.

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特集 外科臨床に必要な漢方治療の知識

術後障害に対する漢方治療

Beneficial effects of Kampo medicine for postoperative complications after digestive organ surgery

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栗田 信浩*5 KURITA Nobuhiro

漢方治療はエビデンスの蓄積と分子生物学的機序解明により適応疾患が拡大されつつある．当科では術後急性期においても，大建中湯，茵蔯蒿湯などの漢方薬剤を積極的に導入している．大建中湯は，腸管運動亢進・腸管血流増加作用により癒着性イレウス改善効果を示し，茵蔯蒿湯は減黄効果とともに肝切除術後の肝細胞保護効果などが期待される．今後，エビデンスのさらなる蓄積とともに術後障害に対しても広く臨床応用されることが期待される．

はじめに

漢方医学は，臨床的有用性が確認されるとともに西洋医学の分子生物学的手法などを用いた基礎的研究により作用機序解明も徐々に進み，今後さらに適応疾患の拡大が予想されている[1]．外科診療における漢方の位置づけとしては，これまではそのほとんどが手術により慢性化した病態に応用されてきたのが実状であった．しかしながら今後は，エビデンスの蓄積とともに術後を含めた急性期疾患においても積極的に適用することで十分に臨床効果が期待できると予想される．

そこで本稿では，消化器外科領域の術後障害に対してわれわれが実践している大建中湯，茵蔯蒿湯，六君子湯などを中心とした漢方治療の適応および有用性を示すメカニズムについて概説する．

I. 消化管手術の術後障害に対する漢方治療

1．大建中湯

大建中湯は乾姜，人参，山椒の3つの生薬に膠飴を加えた方剤であり，とくに山椒は本邦にしかない生薬である．消化管運動亢進，腸管血流増加，腸管過剰運動抑制，抗炎症作用など多彩な効果をもつなど作用機序の解明が進んでいる．実際の臨床現場では癒着性イレウスや麻痺性イレウス，過敏性腸症候群，クローン病などに対して広く使用されている日本独自の漢方方剤である．

1）消化管運動亢進作用

消化管運動亢進には筋層間神経叢におけるセロトニン受容体を介したアセチルコリン遊離作用，

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Key words：大建中湯／茵蔯蒿湯／六君子湯／癒着性イレウス／肝細胞保護

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粘膜層におけるバニロイド受容体を介した直接作用，平滑筋層におけるモチリン分泌作用が関与し，平滑筋の中にある血管神経叢に作用して腸管平滑筋を刺激する[2]．成分別にみると，人参は腸管からの吸収の後に血流を介して消化管運動を亢進させ，山椒と乾姜は吸収後の血流を介さず腸管神経系に直接作用することから投与後30分以内と早期にその効果を発揮する．また，投与部位から肛門側の消化管に対して効果を発揮する．

2）腸管血流増加作用

大建中湯の腹部の冷えの改善効果は腸管運動の亢進のみならず，腸管の血流を介した効果であり，動物実験において消化管内に存在するCGRP(calcitonin gene related peptide：血管拡張因子)を介した作用や[3]，さらにこの血流増加作用がCGRPのみならずRAMP1(CGRP受容体)の発現増加が関与していることも解明されている[4]．

3）抗炎症作用

イレウスが長期に及んだ場合などにもっとも懸念される病態がBacterial translocationである．われわれはラット絶食モデルにおける大建中湯投与効果を検討したところ小腸粘膜組織の萎縮が有意に抑制された(図1)．さらに組織RNAのreal time RT-PCR解析にて炎症性サイトカインの有意な抑制が認められた(図2)[5]．これらの結果は，直接的な腸管の整合性の維持や炎症抑制効果によ

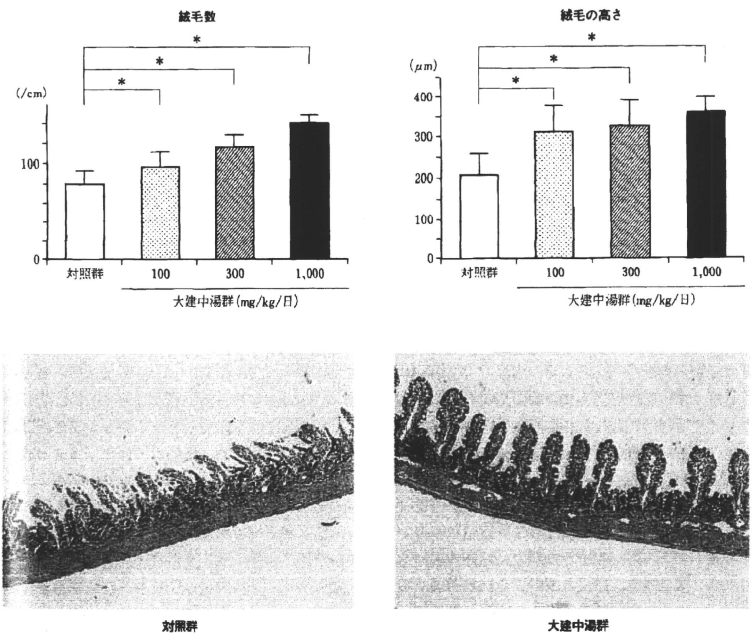

図1　大建中湯の小腸粘膜保護効果(文献5より改変)
＊P<0.05

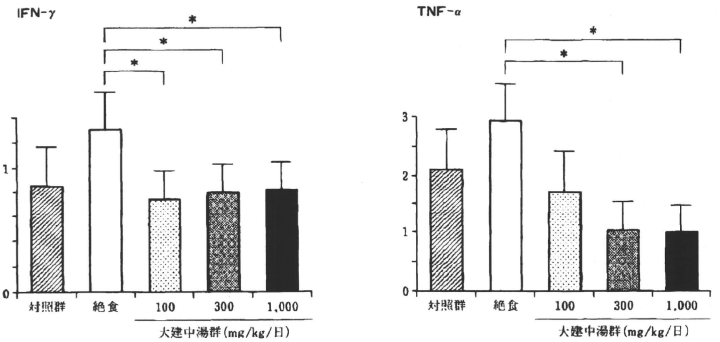

図2 大建中湯の炎症性サイトカイン抑制効果(文献5より改変)
＊P<0.05

りBacterial translocationを予防していることを示唆している.

4）イレウスに対する治療・予防効果

以上のような消化管運動亢進・血流改善作用や抗炎症作用を持つことから，イレウスに対する治療効果は早くから検討されその有用性が報告されてきた[6].

術後癒着性イレウス症例に対し1日7.5～15 gを経口および経鼻胃管，イレウス管から投与を行った群と非投与群の比較を行った報告では，在院日数，経口摂取までの期間が投与群で有意に短縮され，腹部単純X線写真での異常ガスの消失，イレウス管抜去，排便までの期間も改善され，手術までの期間は延長した．本剤投与によって手術までの時間的余裕が生まれ，さらに本剤無効例が手術適応となる可能性も考えられた[7]．大腸癌開腹手術症例に術後第1病日から7.5 gを経口投与した群と非投与群を比較検討した報告では，投与群において術後排ガスまでの期間，術後入院日数が有意に短縮され，在院期間内における腸閉塞の発症も少なかった．また術後1～2病日の早期に投与する方が入院期間短縮に有効であった[8]．さらに，大腸癌手術症例469例を同様に投与群，非投与群に分け，術後在院日数，医療費軽減効果を検討したところ，開腹手術でも腹腔鏡手術であっても術後入院日数は有意に短縮され，とくに腹腔鏡下手術で医療費節減効果がみられた[9]．イレウスとは異なるが，胃全摘術後に空腸嚢間置再建を行った患者に1日15 gを2週間投与した群と非投与群を比較検討すると，投与群で停滞に関する症状が減少し，^{111}Inや^{99m}Tcを用いた排出能試験でも排出の促進がみられ，空腸嚢運動の促進が確認されたとの報告もある[10]．

小児外科領域においても，1日0.1～0.15 g/kgの投与量で術後イレウスや腹部手術後，Hirschsprung病，慢性便秘などのobstructive bowel diseaseの85%に有効であり，本剤が有効であれば投与続行し，無効であれば手術を考慮して精査を進めるとういう治療方針の推奨が報告されている[11].

安価な大建中湯を使用することで，イレウスの予防や腹部術後の回復を早める効果もあり，さらに在院期間を短縮することによって医療経済にも貢献すると考えられる.

2．六君子湯

六君子湯は蒼朮，大棗，人参，陳皮，半夏，甘草，茯苓，生姜という8種の生薬で構成され，消化不良や食欲不振などの上腹部不定愁訴を緩和するとされている．主成分の人参は胃排出促進などの消化管運動亢進作用を持つとされ，胃炎，消化不良，食欲不振，胃痛，嘔吐などに適応される．

実験的検討では粘液亢進作用や，一酸化窒素を介する胃粘膜保護作用が報告され，臨床試験において胃の受容機能不全の改善や胃粘膜血流増加作用，さらに一酸化窒素の前駆物質であるL-arginin含有による胃排出促進作用が報告されている．消化器手術領域においては胃切除術後の消化器症状の改善や胃切除術後逆流性食道炎に対する治療および予防効果が報告されている[12]．

1）胃切除術後の消化器症状の改善

胃切除術後には食事摂取量減少や消化管ホルモン分泌動態の変化がもたらす体重減少をはじめとして，小胃症状や下痢，逆流性食道炎による胸やけなどの多くの愁訴が古くから問題となってきた．六君子湯の持つ消化管運動亢進作用，胃粘膜血流増加作用，グレリン分泌による食欲増進作用によりこれらの症状改善をきたすことが期待される．胃切除術後に悪心，嘔吐，げっぷ，食欲不振などの消化器症状を訴えた症例に六君子湯を投与すると症状の改善がみられ，とくに肥満度がやせ，体表面積が小さい，胃癌全摘症例に高い効果がみられたと報告されている[13]．また，胃切除術後の逆流性食道炎に対しても六君子湯投与が治療および予防に有効であることが示されている．

2）機能温存胃手術との併用効果

幽門側胃切除術後に生じるダンピング症候群を防止するため，幽門輪を温存して胃切除術が行われる場合がある．本術式を行った場合，術後の胃内うっ滞が問題となるが，六君子湯を投与することで胃内容排出亢進作用を示し，とくに消化管排出シンチグラムでは固形物の胃内うっ滞が改善する[14]．

II．肝胆膵手術の術後障害に対する漢方治療

1．大建中湯

消化管手術後のみならず，最近では肝胆膵手術後においてもその有用性が報告されるようになった．Kaihoら[15]は，肝切除例を対象に大建中湯(15 g/日，n=27)とラクツロース(48 g/日，n=31)との投与比較試験を行った．その結果，大建中湯投与群はラクツロース投与群と比較して有意に術後血中アンモニア濃度を低下させるだけでなく，術後排ガス期間の短縮をも認めた．したがって肝切除術後肝障害の予防という観点からも大建中湯の術後早期投与が有用である可能性があると報告している．また，「肝癌切除術施行後の消化管機能異常に対する大建中湯の臨床的効果」に関する多施設二重盲検群間比較試が現在進行中であり，さらなるエビデンスの蓄積が期待される．

1）門脈血流増強作用

われわれは大建中湯の腸管血流増加作用に着目し，ヒトの門脈血流に対する影響について超音波ドプラー法を用いて検討した．健常人では投与後早期(30分以内)に門脈流速，門脈血流ともに非投与群と比較し有意に増加した(図3)[16]．また門脈血流の増加は投与後早期から認められるという結果から大建中湯の門脈血流増加作用は腸管神経系を介した反応によるものと考えられた．肝硬変症例では門脈血流量は投与後早期(30分以内)から有意に増加したものの門脈流速の変動は軽度であり(図3)，その理由として血管径の差，とくに肝においては肝臓の硬さ(類洞の拡張作用)が関与している可能性があると推察している．また肝への神経伝達系が分断されている生体肝移植症例においても門脈流速，門脈血流ともに投与前値と比較して有意に増加することを確認したが，肝硬変症例と同様に比較的軽度で緩徐な反応であったことは，この仮説を支持するものと思われる．さらに注目すべき点は門脈血流の増加が認められるにもかかわらず門脈圧には有意な変動を認めないことである．門脈圧や肝類洞構造への作用については，

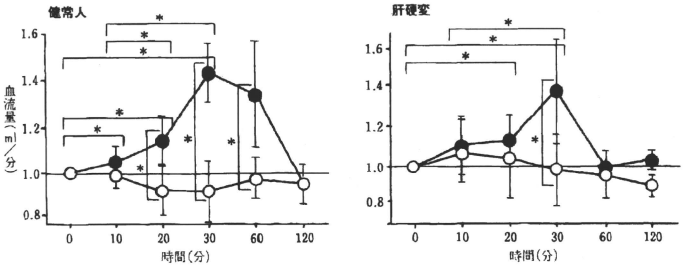

図3 大建中湯の肝血流に対する効果（文献16より改変）
＊P<0.05 ○：対照群，●：大建中湯群

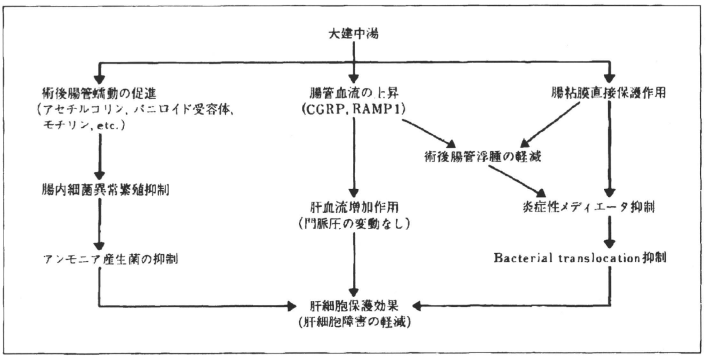

図4 大建中湯の作用機序

今後さらなる機序の解明が必要であるが，少なくとも大建中湯の肝疾患に対する適用拡大への可能性がある。

2）肝保護作用

われわれが推察している大建中湯の肝保護効果の作用機序について図4に示す。大建中湯投与により術後排ガスまでの期間短縮，高アンモニア血症の改善，抗炎症反応の抑制，肝血流増加などの消化器手術急性期における効果が確認された。今後さらに作用機序が解明されることで，とくに肝胆膵外科領域において慢性期・急性期を問わず新たな展開が期待されている。

2．茵蔯蒿湯

茵蔯蒿湯は茵蔯蒿，山梔子，大黄の3つの生薬からなる漢方で，適応疾患として胆汁うっ滞，肝硬変，黄疸，腹水などがあげられ，中でも黄疸に対する有用性が古くから知られている。

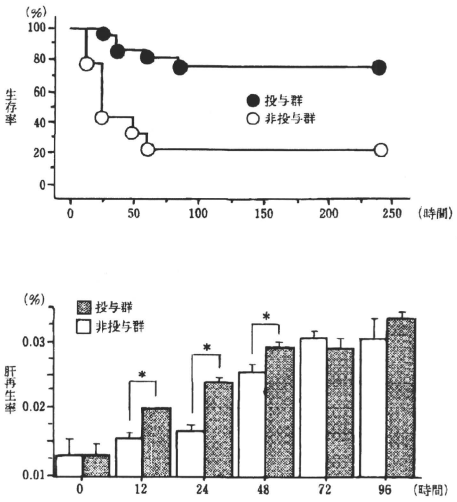

図5 茵蔯蒿湯の肝再生促進効果(文献18より改変)
*P<0.05

その作用機序は茵蔯蒿に含まれる6, 7-dimetylesculetinや山梔子に含まれるgeniposideによる利胆効果と考えられている．geniposideの利胆効果はその活性体であるgenipinによるもので，胆管細胞膜に存在してビリルビントランスポーターとして重要な役割を果たすMRP2などの機能促進による[17]．

実際の茵蔯蒿湯の臨床における使用例としては重症急性肝炎，肝内胆汁うっ滞・黄疸，原発性胆汁性肝硬変，胆道閉鎖症，さらに術後肝障害などに対し有用であると報告され，慢性・急性期ともに黄疸以外の病態においてもその効果が期待される．

大量肝切除術後肝再生に対する効果

肝臓外科領域において，大量肝切除後の肝不全は深刻な合併症の一つである．また生体移植後肝不全の発生にはグラフトサイズが重要な因子であり，過小グラフト症候群の克服は重要な課題である．

われわれは茵蔯蒿湯の大量肝切除術後肝障害に対する効果についてラット実験モデルにおける検討を行ったところ茵蔯蒿湯投与により大量肝切除術後生存率の改善とともに肝再生率の上昇(図5)，肝障害軽減作用を認めた．また肝細胞保護効果を有するHemoxigenaze-1(HO-1)の誘導とともに肝繊維化や炎症誘導メディエータである肝星細胞(α-SMA)の抑制効果も確認した(図6)．さらにmicroarrayによる解析では，茵蔯蒿湯の投与により肝保護効果を有するHeat shock protein familyや肝再生促進因子であるFolliatatinの発現増強とともに炎症性メディエータであるTNF familyの発現抑制を確認した．これらの検討結果から茵蔯蒿湯は，肝切除術後急性期においても肝保護効果のみならず抗炎症効果を有し術後

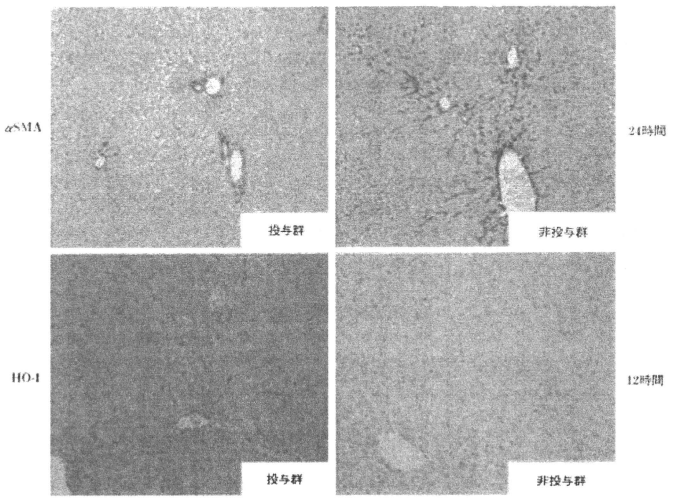

図6 大量肝切除に対する茵蔯蒿湯の効果(文献18より改変)
αSMA：α smooth muscle actin HO-1：Heme oxygenase-1

肝不全を防止することが確認された[18]．これらの知見を考慮すると術前術後を含めた急性期肝疾患への応用への期待が今後さらに高まるといえる．とくに大量肝切除術後は肝再生に伴う黄疸の遷延化が認められることがあるが，黄疸の改善だけでなく肝保護効果さらには抗炎症効果も得ることができ術後肝不全の防止に繋がると考えている．

3．六君子湯

膵頭十二指腸切除術腹部不定愁訴に対する効果

上述のごとく消化管手術，とくに胃切除後における六君子湯の効果はすでに確認されている．われわれはその胃内容うっ滞の改善効果に着目し，膵頭十二指腸切除術後における遅延性胃内容停滞(delayed gastric empty：DGE)に対する六君子湯の有用性について検討した．投与前後の活性化グレリン値とともに，消化器疾患のQOL評価には消化器症状全般について患者のQOLを評価する Gastrointestinal Symptom Rating Scale (GSRS)を用いスコア変化を検討した．GSRSは15の質問に対する回答から酸逆流，腹痛，消化不良，下痢，便秘の5つの消化器症状のサブグループに分類し，患者のQOLを測定するものである．活性化グレリンは，投与前は9例すべてが検出限界以下であったのに対し投与後は4例で検出可能な値まで上昇しており，さらにGSRSスコアの有意な改善も認め，六君子湯のDGEに対する有用性が示唆された(図7)．

おわりに

消化器外科領域における術後障害に対する漢方治療の応用において，とくに有用と考えられる大建中湯，茵蔯蒿湯，六君子湯を中心に概説した．今後も基礎的・臨床的研究が進むにつれて，さらに適応疾患が拡大することが予想される．最近では漢方医学がこれらのエビデンスをもとに医学教

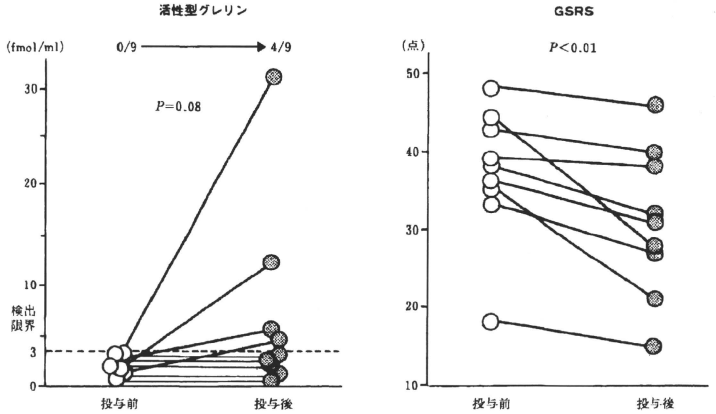

図7　膵頭十二指腸切除術後の消化器症状に対する六君子湯の投与効果

育にも導入され，今後の医学・医療において必須のものとなりつつある。そのような中で慢性期代替医療と言われた漢方医療を積極的に外科医療に導入していくことはまさに自然な流れといえる。

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特集 消化器疾患と漢方

Gastrointestinal Research

肝・胆・膵手術と漢方

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Summary
最近，漢方薬剤は分子生物学的作用機序の解明とエビデンスの蓄積が著しく，その適応疾患の拡大が期待されている．当科では術後急性期において漢方薬剤を積極的に導入し，とくに肝疾患においては術直後から大建中湯と茵蔯蒿湯を頻用している．大建中湯は肝切除術後高アンモニア血症の改善や門脈血流の増加，Bacterial translocation の抑制が見込まれ，茵蔯蒿湯においては減黄効果とともに，肝切除術後の炎症反応の抑制や肝細胞保護効果，肝再生増強効果が期待される．さらに胆・膵疾患における幽門輪温存膵頭十二指腸切除術後の消化器症状に対しても，六君子湯を投与することで改善効果が認められている．今後，エビデンスの蓄積とともに漢方医療を積極的に外科医療に導入していくことも必要と思われる．

Key words
大建中湯　茵蔯蒿湯　六君子湯　肝胆膵手術　漢方

はじめに

漢方はおもに内科的慢性疾患に使用されていたが，その理由の一つとして外科的治療概念の乏しい古代中国により体系化されてきたことがあげられる．また西洋医学と比較してその作用機序が明らかでなかったため慢性疾患における代替医療としての役割しか果たしてこなかったのが現状である．しかし，最近になり西洋医学の基礎的研究の手法を用いることによって，その臨床における有用性や分子生物学的な作用機序の解明が進み，今後さらに適応疾患の拡大が考えられる．

このようななかで外科医療における漢方の位置づけとして，現在までそのほとんどが手術により慢性化した病態などに応用されているのが実状であるが，今後，エビデンスの蓄積とともに術後を含めた急性期疾患においても，積極的に適用すべきであり，術後周術期を含んだ外科領域への適応拡大が期待される．

今回，肝・胆・膵疾患において重要と考え，実際の臨床の場で実践している大建中湯（ダイケンチュウトウ），茵蔯蒿湯（インチンコウトウ），六君子湯（リックンシトウ）を中心に肝・胆・膵疾患への適用・応用とメカニズムについて概説する．

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