CONCLUSIONS

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

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The clinical significance of SWI/SNF complex in pancreatic cancer

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Received September 21, 2012; Accepted November 5, 2012

DOI: 10.3892/ijo.2012.1723

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

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

Introduction

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

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

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

Materials and methods

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

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

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

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

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

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

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

Table I. The clinicopathological characteristics of all patients.

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

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

Results

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

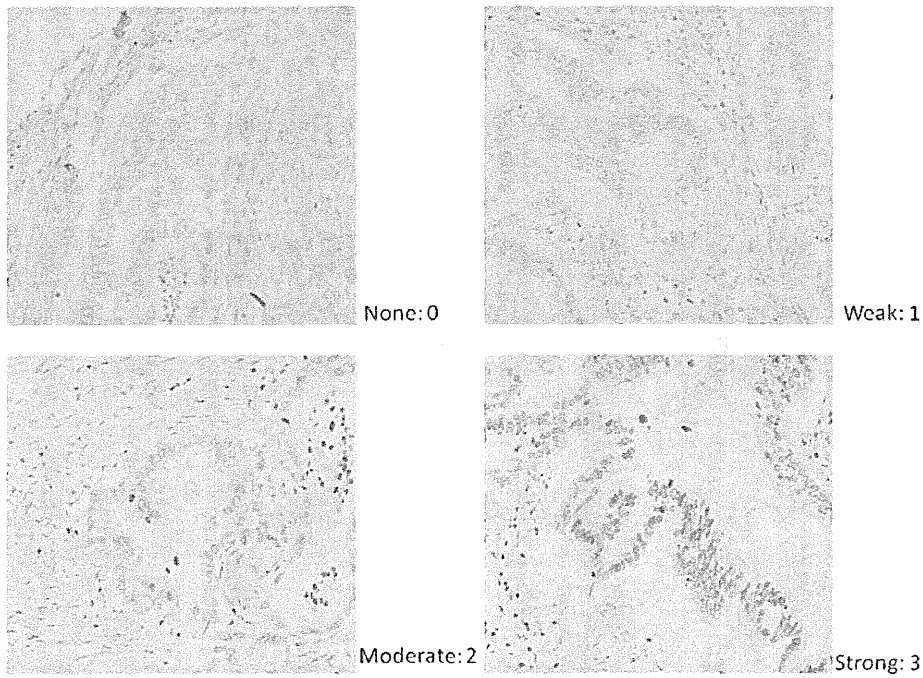

Figure 1. Histoscore (H-score) was calculated by a semi-quantitative assessment of both the intensity of staining (graded as: 0, non-staining; 1, weak; 2, median; or 3, strong using adjacent normal mucosa as the median) and the percentage of positive cells. The range of possible scores was from 0 to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

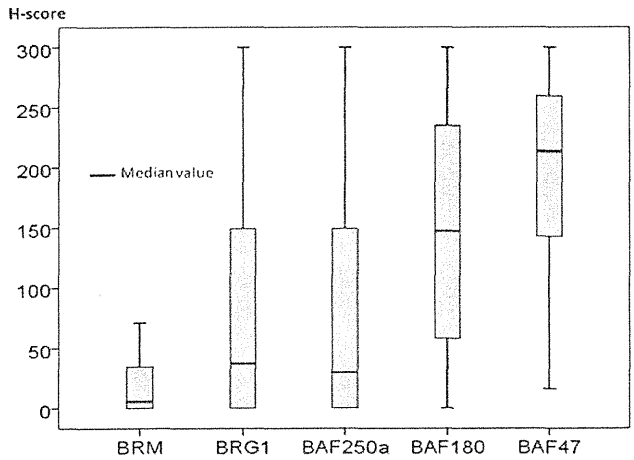

Figure 2. The distribution of the H-score is shown in the box plot. The horizontal bar shows the median value of each score.

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

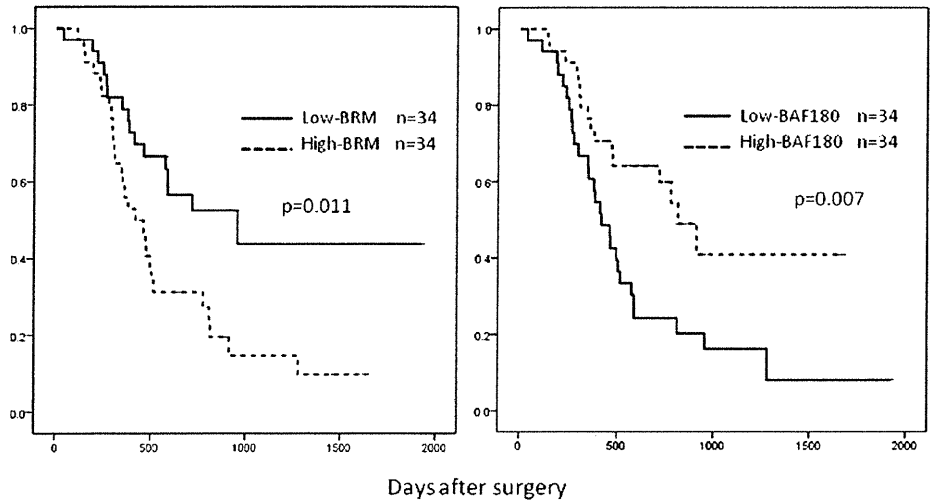

Figure 3. The survival curves were compared by Kaplan-Maier method by the expression level of BRM and BAF180. The statistical significance was evaluated using log-rank test.

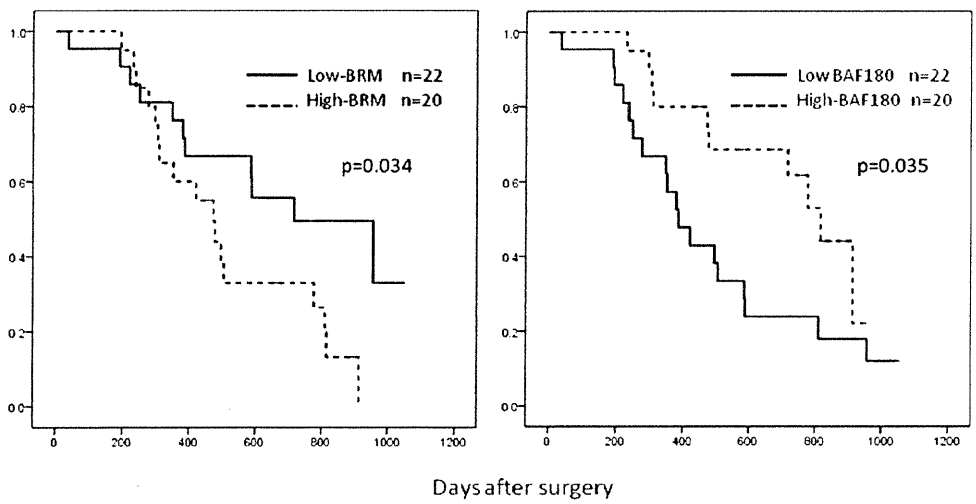

Figure 4. The survival curves of patients with adjuvant gemcitabine were compared by Kaplan-Maier method by the expression level of BRM and BAF180. The statistical significance was evaluated using log-rank test.

organ metastasis, lymphatic invasion, and stage IV disease. Stage IV disease was also correlated to high BRG1, which is reported to have similar biological function as BRM. On the other hand, better clinicopathological features were related to high BAF expression. High BAF180 was related to smaller tumor size, and high BAF47 was associated with negative lymphatic invasion.

In addition, our multivariate analysis revealed both high BRM and low BAF180 were independent prognostic indicators for poor survival, whereas the expression level of BRG1, BAF250a, and BAF47 were not related to overall survival.

As a next step, we investigated the prognostic significance of these factors in the patients with adjuvant gemcitabine. Gemcitabine remains standard therapy in the adjuvant and palliative settings for pancreatic cancer (29,30). However, the response rate of gemcitabine is very low, with only 18% of 1-year survival rate (31). Developing a novel biomarker, which predicts the response for gemcitabine, is urgently needed. In the analysis of the patients with gemcitabine, we reached the same result; both high BRM and low BAF180 were independent prognostic indicators for poor survival.

A previous study showed that BRM or BRG1 is lost in 10-20% of the bladder, colon, breast, esophageal, pancreatic and ovarian cancers by immunohistochemical staining of tissue microarrays (32). Another study reported BRM was lost in approximately 15-20% of primary non-small lung cancers, and silencing of BRM was a prognostic factor for poor outcome (33,34). Although BRM is supposed to be involved in many biological functions, these data showed BRM-containing complexes (BRM/BAF) as tumor suppressor in cancer tissue.

It is also reported that BRM has a role in transcription of CD44 (35), which is important in the process of tumor-endothelium interactions, cell migration, cell adhesion, tumor progression and metastasis (36).

Our result showed that the patient with high BRM had a significantly worse survival than those without (5-year OS:

Table V. Multivariate analysis for overall survival in patients with adjuvant gemcitabine.

Factors	HR (95% CI)	p-value
Age		0.002
<65	1.0	
≥65	0.227 (0.089-0.580)	
Tumor size (cm)		0.280
<4	1.0	
≥4	0.593 (0.230-1.531)	
Histology		0.267
Well/Mod	1.0	
Poor	1.907 (0.610-5.964)	
M		0.923
M0	1.0	
M1	0.947 (0.315-2.847)	
Curability of surgery		0.784
R0	1.0	
R1	1.145 (0.433-3.029)	
BRM		0.017
Low	1.0	
High	3.411 (1.251-9.305)	
BAF180		0.016
Low	1.0	
High	0.336 (0.138-0.819)	

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

9.8 vs. 43.8%, p=0.009), suggesting BRM/BAF in pancreatic cancer may contribute to tumor progression.

We also revealed the significant relationship between high BAF180 expression and smaller-sized tumor, and identified BAF180 as an independent prognostic factor for better survival in pancreatic cancer.

BAF180 maps to the 3p12 region (37) where allele loss is frequent and homozygous deletion have been detected in lung and breast cancer cell lines (38,39). Thus, genes located on this region have been thought as candidates for tumor suppressors. Actually, it is reported that BAF180 mutation is associated with carcinogenesis of breast cancer, and BAF180 suppresses tumorigenesis through its ability to regulate p21 (40), which controls the cell cycle (41). Recent research also clarified BAF180 mutation in clear cell renal cell carcinoma (42). These results suggest the idea that BAF180-containing complexes (PBAF) suppress tumor progression, which does not contradict our present results.

BAF250a-containing SWI/SNF complexes (BRG1/BAF) are reported to have different structure and biological properties from PBAF (43,44). A previous study showed that BAF250a was deleted in as many as 30% of renal cell carcinoma and 10% of breast carcinoma (19,45). These results lead to the concept that BRG1/BAF appear to have antagonistic effect on cell cycle progression (46). However, our data did not show the relationship of BAF250a expression to clinicopathological features or overall survival in pancreatic cancer.

Based on this study, we reached the conclusion that high BRM, and low BAF180 are useful biomarker not only for the patients with curative resection, but also for those with adjuvant gemcitabine. Future investigation into biological functions of SWI/SNF components could lead to better management in pancreatic cancer.

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Accepted 16 August 2012
Available online 6 September 2012

Available online at www.sciencedirect.com

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Accepted 16 August 2012
Available online 6 September 2012

Abstract

Background: To search for biomarkers identifying pancreatic cancer patients likely to benefit from adjuvant gemcitabine chemotherapy, we investigated the status of several histone modifications in pancreatic tumors and their relationship to clinicopathological features and outcomes.

Methods: Sixty one pancreatic cancer patients, primarily treated by surgical removal of tumors, were involved in the study. Thirty patients completed postoperative adjuvant gemcitabine, and in 31 it was discontinued. Tumor specimens were examined using immunohistochemistry for di- and tri-methylation of histone H3 lysine 4 (H3K4me2 and H3K4me3), dimethylation and acetylation of histone H3 lysine 9 (H3K9me2 and H3K9ac), and acetylation of histone H3 lysine 18 (H3K18ac). Positive tumor staining for each histone modification was used to classify patients into low- and high-staining groups, which were examined for relationships to clinicopathological features and clinical outcomes.

Results: High expression of H3K4me3 was related to the well and moderately differentiated tumor histological type ($p = 0.012$) and low expression of H3K4me2 was related to the presence of perineural invasion ($p = 0.007$). No cellular histone modifications were associated with overall or disease-free survival of patients as a whole. In the subgroup analyses, a low level of H3K4me2 was significantly associated with worse disease free survival in patients that completed adjuvant gemcitabine ($p = 0.0239$). Univariate and multivariate hazard models also indicated that a low level of H3K4me2 was a significant independent predictor of disease-free survival ($p = 0.007$).

Conclusion: H3K4me2 was found to be a predictor of response to adjuvant gemcitabine in Asian patients with pancreatic cancer.

Keywords: Histone modification; Pancreatic cancer; Gemcitabine

Introduction

Pancreatic cancer remains an important cause of death in many nations.[1] Surgical removal of tumors is the only curative approach, and gemcitabine chemotherapy is the standard treatment after surgery.[2] Prognosis after resection, even followed by gemcitabine, remains extremely poor. Thus, it is important to identify specific biomarkers of outcomes in order to select patients who could be recommended for more aggressive treatment.

Posttranslational histone modifications of chromatin, including methylation, acetylation, phosphorylation, sumoylation and ubiquitination, play critical roles in creating transcriptional activation and repression patterns, in part through the regulation of chromatin structure.[3] Modifications to histone as a result of methylation, which usually occurs at lysine or arginine residues, are generally associated with gene inactivation[4,5] or silencing.[6–8] On the other hand, acetylation of histone, which mostly occurs at lysine residues in the N-terminal domains, is known to be associated with transcriptional activation.[9–11]

Recent studies have indicated that patterns of certain histone modifications, not at the level of each specific

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0748-7983/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ejso.2012.08.008

gene, but at the level of the individual cell as a whole, are associated with the clinicopathological features and outcomes of several tumor types in humans, including prostate, kidney, lung, gastric, colorectal, ovarian, breast and pancreatic.[6,12–17] Two studies on pancreatic cancers have demonstrated that low cellular levels of methylation of histone H3 at lysine 4 (H3K4), lysine 9 (H3K9) or lysine 27 (H3K27), or in the acetylation of H3 at lysine 18 (H3K18) were independent predictors of poor patient survival among the Caucasian population.[16,17] In particular, low cellular levels of dimethyl-H3K4 (H3K4me2) and dimethyl-H3K9 (H3K9me2) were predictive of survival specifically for those patients receiving adjuvant chemotherapy with fluorouracil, but not with gemcitabine.[17]

In a randomized clinical trial, gemcitabine was found to provide a survival advantage over fluorouracil in addition to symptom-relief in patients with advanced pancreatic cancer.[2] Recent studies have revealed that gemcitabine exhibits ethnic differences in terms of efficacy[18] and adverse reactions, associated in part with cytidine deaminase (CDA) gene polymorphism in the Asian population.[19] The aim of the present study was to determine the patterns of histone modifications in pancreatic cancer among the Japanese population, and to investigate the association between these patterns and clinicopathological features and the benefits of postoperative gemcitabine chemotherapy.

Materials and methods

Patients and samples

This study involved the retrospective analysis of 61 patients with surgically removed pancreatic cancer. All of the patients had undergone curative radical resection for primary pancreatic adenocarcinoma at Kanagawa Cancer Center, Yokohama, Japan, between January 2006 and December 2009. We offered postoperative gemcitabine chemotherapy to all patients. Each patient received adjuvant chemotherapy using one of the following protocols: the gemcitabine standard protocol (gemcitabine 1000 mg/m^2, days 1, 8, and 15, every 4 weeks for 6 months) or the gemcitabine biweekly protocol (gemcitabine 1000 mg/m^2, biweekly for 6 months). Although administration was discontinued in 31 patients, 30 patients completed treatment with gemcitabine at a dose of 12 g, which is considered to be a sufficient dose for adjuvant chemotherapy. Informed consent was obtained from each patient. The Ethics Committees of Kanagawa Cancer Center approved the protocol before initiation of the study. None of the patients had any other malignancies.

Immunohistochemistry

Microarrays consisting of two cores, each 2 mm in diameter, were prepared from formalin-fixed paraffin-embedded tissue blocks of surgically removed primary tumor.

Immunohistochemical staining was performed using commercially available polyclonal rabbit anti-histone antibodies raised against dimethyl histone H3 lysine 9 (H3K9me2), acetyl histone H3 lysine 9 (H3K9ac), dimethyl histone H3 lysine 4 (H3K4me2), trimethyl histone H3 lysine 4 (H3K4me3) and acetyl histone H3 lysine 18 (H3K18ac) (Cell Signaling Technology Inc., Daners, MA, USA).

Tissue microarray sections were deparaffinized with xylene and rehydrated with a graded series of aqueous ethanol. For antigen retrieval, slides were placed in Tris/EDTA pH9.0 buffer and autoclaved at 121 °C for 15 min. Endogenous peroxidases were blocked with 3% hydrogen peroxide solution. Then the sections were incubated with primary rabbit anti-histone polyclonal antibodies for 60 min at room temperature at the following dilutions: anti-H3K9me2, H3K9ac, H3K4me3, H3K18ac at 1:300 and anti-H3K4me2 at 1:600. Thereafter, the sections were treated with HRP polymer kit (Nichirei Biosciences, Tokyo, Japan) for signal amplification. Diaminobenzidine-hydrogen peroxide was used as the chromogen, and counterstained with hematoxylin.

Determination of histone modifications score

Immunohistochemical scoring was undertaken using the modified Histo-score (H-score),[11] which involves semi quantitative assessment of both the intensity of staining (graded as non staining: 0, weak: 1, moderate: 2, strong: 3, adjacent normal pancreatic exocrine cells were graded as the median) and the percentage of positive cells (0–100). The range of possible scores was 0–300, enabling us to explore the rationalization of our patients into biologically relevant groups depending on different levels of detection, which could potentially be missed using simpler scoring methods. Tumor samples with an H-score of <150 for individual chromatin marks were designated as low detection, whereas scores of $\geqq 150$ were designated as high detection.

Statistical analysis

The relationship between histone modification levels and potential explanatory variables, including age, gender, location, tumor size, histological type, depth of invasion, lymph node metastasis, location, lymphatic invasion, venous invasion, perineural invasion, serum CEA and CA19-9 concentrations, was evaluated using the chi-square test. The postoperative survival rate and disease-free survival rate were analyzed using the Kaplan–Meier method, and differences in survival rates were assessed using the log-rank test. A Cox proportional-hazard model was used for univariate and multivariate analyses. Differences were considered as significant when the p value was <0.05. Each statistical analysis was performed using the Dr. SPSS II software program, version 11.0.1J for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Patients characteristics

All patient characteristics are detailed in Table 1 with histone modification levels. Of all 61 patients in the present study, 35 were male and 26 were female, and the median age was 64 (44–84) years. Pancreaticoduodenectomy was performed in 37 patients; 16 patients underwent distal pancreatomy and eight patients underwent total pancreatomy. The median size of the resected tumor was 40 (10–95) mm. The median serum CEA concentrations were 3.7 (0.7–70.2) ng/ml, and the median serum CA19-9 concentrations were 270 (2–14794) ng/ml. TNM stages, based on the UICC 7th edition, were IB:2, IIA:13, IIB:28, III:18.

Within a median follow-up duration of 14.4 (3.8–58.8) months, recurrences were found in 44 patients and deaths occurred in 39 patients.

Immunohistochemistry and H-score distributions

Representative staining for each of the five histone modifications is shown in Fig. 1. Only nuclear staining was regarded as positive, and cases were scored for each mark using a modified H-score as described in the Materials and methods. Histograms showing the distribution of H-scores plotted against the number of cases for each histone modifications are shown in Fig. 2. The median value (range) for each H-score was as follows: H3K9me2, 158 (5–300); H3K9ac, 140 (0–286); H3K4me2, 142 (0–222); H3K4me3, 160 (48–288); H3K18ac, 162 (58–300). H-scores of H3K9me2 and H3K18ac were almost exclusively accumulated in the range 151–200. Although scores in the range 151–200 were also most frequently observed in H3K9ac, H3K4me2 and H3K4me3, scores in the range 51–100 were the second most frequent in these modifications. Based on the finding that the cut off value for the H-score using ROC curve analysis was almost identical to the median value, the expression level of the histone modifications was categorized as being low if they were <150 or high if they were $\geqq 150$, to keep the scores clear and concise.

Relationship between the histone modifications and clinicopathological features

The relationships between the expression levels of histone modifications and the patients' clinicopathological

Table 1
Relationship between the expression of histone modifications and the clinicopathological features.

Variables/ categories	H3K9me2			H3K9ac			H3K4me2			H3K4me3			H3K18ac		
	Low	High	*p* Value	Low	High	*p* Value	Low	High	*p* Value	Low	High	*p* Value	Low	High	*p* Value
	$n = 29$	$n = 32$		$n = 35$	$n = 26$		$n = 36$	$n = 25$		$n = 14$	$n = 47$		$n = 15$	$n = 46$	
Location															
Head	20	24	0.600	24	20	0.472	27	17	0.549	10	34	0.947	9	35	0.228
Body/tail	9	8		11	6		9	8		4	13		6	11	
Tumor size															
≦2 cm	1	5	0.111	3	3	0.700	1	5	**0.026***	0	6	0.159	1	5	0.635
>2 cm	28	27		32	23		35	20		14	41		14	41	
Histological type															
Well, mod	21	31	**0.007***	28	24	0.180	31	21	0.819	9	43	**0.012***	12	40	0.509
Por, others	8	1		7	2		5	4		5	4		3	6	
Depth of invasion															
T1, T2	2	1	0.496	1	2	0.388	1	2	0.354	0	3	0.332	1	2	0.718
T3, T4	27	31		34	24		35	23		14	44		14	44	
Lymph node metastasis															
Absent	7	8	0.938	12	3	**0.041***	7	8	0.263	3	12	0.754	2	13	0.244
Present	22	24		23	23		29	17		11	35		13	33	
Venous invasion															
Absent	9	11	0.933	11	9	0.770	11	9	0.628	4	16	0.630	4	16	0.630
Present	18	21		23	16		24	15		10	29		10	29	
Perineural invasion															
Absent	9	7	0.409	8	8	0.47	5	11	**0.007***	5	11	0.297	6	10	0.194
Present	19	24		26	17		30	13		8	35		9	34	
CA19-9															
Normal	4	7	0.42	3	8	0.056	4	7	**0.047***	2	9	0.813	1	10	0.284
Abnormal	21	21		25	17		29	13		9	33		10	32	

Well: well differentiated, Mod: moderately differentiated, Por: poorly differentiated.
Bold values represent less than 0.05.

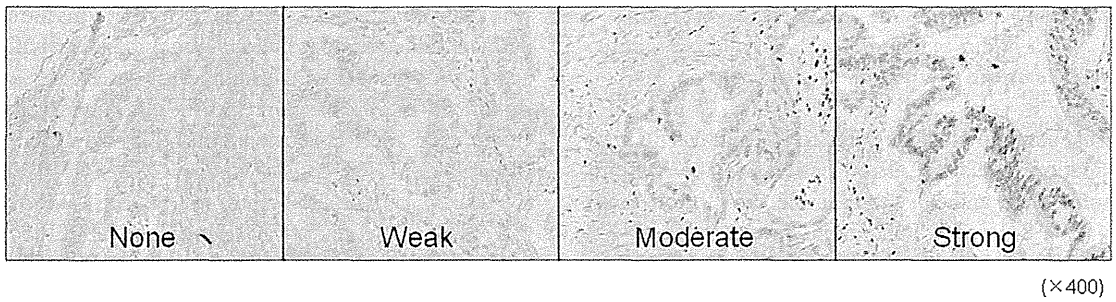

Figure 1. Representative examples of H3K4me2 immunohistochemical staining in pancreatic cancer tissues. Demonstrative images for each criterion are shown. Scale-bars: 100 μm.

features were then examined (Table 1). The low H3K9me2 expression group was significantly associated with the group of poorly differentiated adenocarcinomas or histological types other than adenocarcinoma ($p = 0.007$). In contrast, the high H3K4me3 expression group was significantly associated with the group of well and moderately differentiated adenocarcinomas ($p = 0.012$). Other modifications were not associated with tumor histological type. The low H3K4me2 expression group was significantly associated with the presence of perineural invasion ($p = 0.007$) and elevated serum CA19-9 concentrations ($p = 0.047$). The high H3K4me2 expression group was associated with smaller tumor size ($p = 0.026$). The low H3K9ac expression group was related to the absence of lymph node metastasis ($p = 0.041$). Histone modifications were unrelated to age, gender, tumor location, lymphatic invasion, venous invasion, depth and serum CEA concentrations.

Relationship of histone modifications to patient overall and disease-free survival

We compared the overall and disease-free survival rates among the cases with different levels of histone modification using the log-rank test. The overall and disease-free survival (DFS) rates did not appear to differ according to H3K9me2, H3K9ac, H3K4me2, H3K4me3 or H3K18ac status (data not shown).

Histone modification levels and adjuvant gemcitabine chemotherapy

We next examined whether histone levels were able to predict patient response to gemcitabine chemotherapy. We stratified patients on the basis of postoperative therapy; the patients in group A received gemcitabine chemotherapy

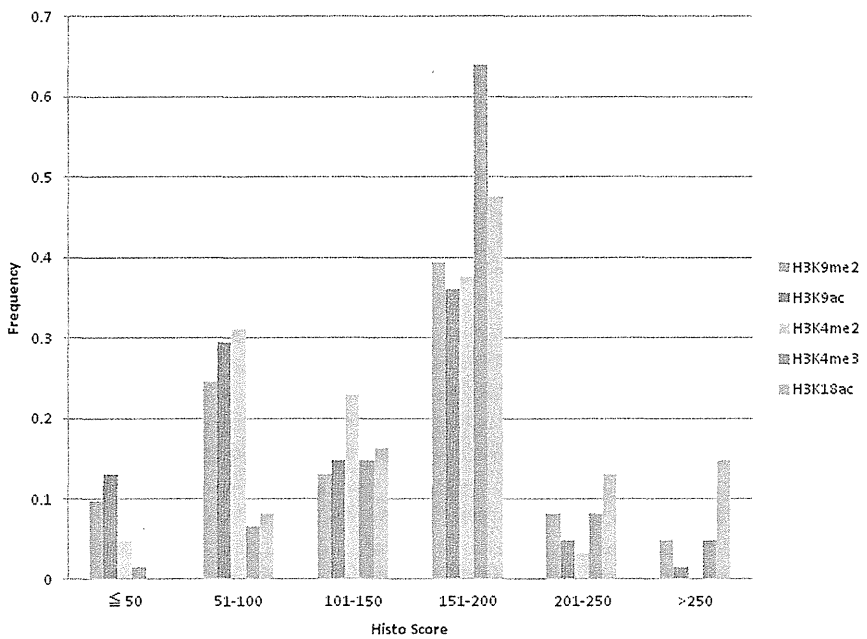

Figure 2. Histograms showing the distribution of H-scores plotted against the number of cases exhibiting the histone modifications.

at a dose of 12 g and those group B did not received chemotherapy or did not achieved a dose of 12 g. In group B, 10 patients did not start gemcitabine because of their unwillingness to undergo treatment, and the remaining 21 patients commenced gemcitabine but abandoned it during the course of treatment, generally due to the adverse effects. Evaluation of clinicopathological factor groups A and B using the chi-square test, revealed that only the presence of lymph node metastasis was significantly associated with Group B ($p = 0.031$). Using Kaplan–Meier survival analysis it was found that the low H3K4me2 expression group was significantly associated with the worse DFS in group A that received the full-dose of gemcitabine (Fig. 3). Both univariate and multivariate hazard models also indicated that the low H3K4me2 expression group was a significant independent predictor of DFS ($p = 0.007$) (Table 2).

Discussion

We used immunohistochemistry to evaluate the modification patterns of five different histone residues at the cellular level in 61 surgically removed pancreatic tumors and examined the relationship between histone modifications and patient clinicopathological features and outcomes.

Relationship between the histone modifications and clinicopathological features

A low level of cellular methylation of H3K4 was associated with perineural invasion and elevated serum CA19-9 concentrations, and a low level of cellular methylation of H3K9 was associated with the histology of the group, including poorly differentiated adenocarcinoma and tumors other than adenocarcinoma. In contrast, a high cellular level of methylation of H3K4 was associated with smaller tumor size and a well or moderately differentiated adenocarcinoma histology. Although different effects of methylation on gene transcription, namely activation or repression, have been reported for H3K4 (activation),[17] H3K9 (activation/repression)[5,7,8] or H3K27 (repression),[3] cellular methylation levels of histone H3 were generally considered to be associated with unfavorable clinicopathological characteristics in our study. This feature was consistent with the preceding two reported studies on pancreatic cancer.[16,17] A similar association has been reported in ovarian and breast cancers.[16] In stage I non-small-cell lung cancer (NSCLC) patients, a high level of H3K4me2 has been reported to be associated with the best survival rates,[13] which can be considered as a similar trend to that found in pancreatic cancer. In contrast, gastric adenocarcinomas with a high level of H3K9me3 were associated with unfavorable characteristics such as higher T stage, nodal metastasis and recurrence.[14] Cellular histone methylation levels may have different impacts on different tumor types, and also on the location of methylated lysine residues.

Impact of histone modification levels on disease free survival

In the present study, we did not find any association between a low cellular level of H3K4me2, H3K4me3 or H3K9me2 and the overall and disease-free survival rates of patients, in spite of a positive correlation with unfavorable characteristics. Because previous papers have revealed a correlation with poorer survival,[16,17] we may need a larger

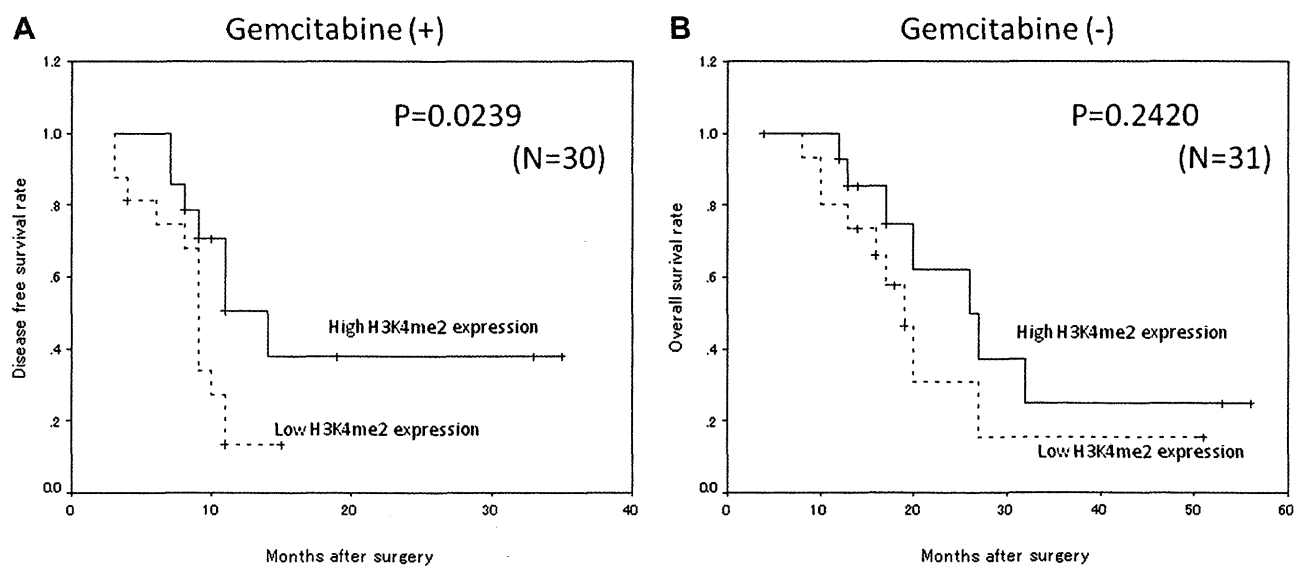

Figure 3. Disease-free survival according to postoperative chemotherapy. The low H3K4me2 expression group was significantly associated with the worse DFS in group A.

Table 2
Univariate and multivariate Cox regression analyses of factors affecting disease-free survival.

Variables/categories	*n*	Univariate			Multivariate		
		HR	95% CI	*p* Value	HR	95% CI	*p* Value
Location							
Head	21	1	0.874–7.061	0.088			
Body/tail	9	0.248					
Tumor size							
≦2 cm	3	1	0.300–17.02	0.428			
>2 cm	27	2.261					
Lymph node metastasis							
Absent	11	1	0.678–4.665	0.242			
Present	19	1.778					
H3K4me2 expression							
Low	16	1	0.132–0.904	**0.030***	1	0.038–0.600	**0.007***
High	14	0.346			0.151		
Histological type							
Well, mod	24	1	0.370–4.357	0.703			
Por, others	6	1.271					
Vascular invasion							
Absent	12	1	0.446–2.915	0.783			
Present	17	1.141					
Lymphatic invasion							
Absent	9	1	1.039–19.74	**0.044***	1	0.568–11.739	0.220
Present	21	4.498			2.582		
Perineural invasion							
Absent	9	1	0.613–5.525	0.277			
Present	21	1.841					

CI: confidence interval, Well: well differentiated, Mod: moderately differentiated, Por: poorly differentiated.
Bold values represent less than 0.05.

cohort to clarify this issue. However, in a subgroup analysis, we found that a low level of H3K4me2 was associated with worse disease free survival in patients receiving adjuvant gemcitabine. This result was different from that reported in a preceding study by Manuyakorn et al.[17] that indicated a positive association between a low level of H3K4me2 and disease free survival only for those patients that had received adjuvant fluorouracil, but not for those that had received gemcitabine. Differences in drug efficacy and toxicity have been reported between Asians and Caucasians.[20] Polymorphic variations in genes involved in gemcitabine pharmacology could be a cause of these differences.[21] Actually, Ross et al.[18] found significant differences in the distribution of genotypes between healthy Asians and Caucasians in 13/19 loci in the genes involved in gemcitabine pharmacology. It has been further reported that the variant of the CDA gene, involved in gemcitabine detoxification,[22,23] was associated with response rate and time to progression, and that the variation of the SLC28A1 gene, a gemcitabine transporter,[24,25] was associated with hematologic toxicity in patients with NSCLC receiving gemcitabine-based treatment.[18] Ethnic genetic background could be responsible for the difference in response to adjuvant gemcitabine between previous studies involving the Caucasian population and the present study involving the Japanese population.

Conclusion

We indicated that H3K4me2 at the cellular level might be useful in identifying pancreatic cancer patients who would be likely to derive benefit from adjuvant gemcitabine. Although our study had several limitations including the small sample size and its retrospective nature, we believe that the results obtained are meaningful, and should be strengthened by adequately powered future studies.

Conflict of interest statement

The authors declare that they have no potential conflict of interest.

Acknowledgment

This study was partially supported by grants from Kanagawa Prefectural Hospitals Cancer Fund.

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JJCO Japanese Journal of Clinical Oncology

Reprinted from

Jpn J Clin Oncol 2012;42(9)800–806
doi:10.1093/jjco/hys102
Advance Access Publication 23 July 2012

A Retrospective Study of S-1 Monotherapy as Second-line Treatment for Patients with Advanced Biliary Tract Cancer

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Received January 30, 2012; accepted June 7, 2012

Objective: Gemcitabine has been widely used, and cisplatin plus gemcitabine is considered as standard first-line chemotherapy for patients with advanced biliary tract cancer. However, no standard therapy was established following the progression to gemcitabine-containing first-line therapy. As S-1 monotherapy as second-line chemotherapy is still not well known in a practical setting this study aimed to clarify its efficacy and safety.
Methods: We retrospectively reviewed 55 consecutive patients who received S-1 monotherapy as second-line chemotherapy after failure of a gemcitabine-containing regimen at our institution from September 2007 to March 2011. The inclusion criteria were preserved organ function and an Eastern Cooperative Oncology Group performance status of 0–2 and without massive ascites or pleural effusion. S-1 was administered orally twice a day at a dose of 40 mg/m^2 for 28 days, followed by 14 days of rest.
Results: Fifty-one patients were selected for this analysis. The overall response rate was 4.0% and the disease control rate was 38.0%. The median survival time was 6.0 months and the median progression-free survival was 2.3 months. Adverse events were generally mild, and treatment-related death did not occur. In the subgroup analysis, overall survival was significantly shorter in the patients with peritoneal dissemination and those who had shown no response to the first-line chemotherapy ($P = 0.033$ and 0.023, respectively).
Conclusions: S-1 monotherapy as the second-line chemotherapy for patients with gemcitabine-refractory advanced biliary tract cancer is also feasible in a practical setting and its efficacy is almost the same as in the previous prospective study.

Key words: S-1 – biliary tract cancer – second-line – gemcitabine refractory

INTRODUCTION

Biliary tract refers to all routes that bile juice passes through from hepatocytes to the duodenum, including intrahepatic bile duct, extrahepatic bile duct, gall bladder and ampulla of Vater. Therefore, biliary tract cancer (BTC) includes intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, gallbladder carcinoma and ampullary carcinoma. Sometimes, intrahepatic cholangiocarcinoma is classified as primary liver cancer by UICC (1) and Japanese classification (2), but it is more often classified as BTC because of its development, as well as pathological and clinical features.

BTC is not a common disease throughout the world; however, it is more commonly encountered in East Asia and Latin America than any other countries (3). Furthermore, it is the sixth leading cause of cancer-related death in Japan.

They are usually found in unresectable stage; however, resection surgery is the only way to cure BTC. Moreover, recurrence after curative surgery is common because BTC has high malignant potential and propensity to metastasize. Therefore, systemic chemotherapy is important for the treatment of BTC. Gemcitabine (GEM) has shown efficacy and safety for advanced BTC in many reports (4–6). GEM is considered the key drug for the treatment of advanced BTC, and GEM monotherapy was recognized as a community standard in Japan until 2010. In 2010, the results of the Phase III study of cisplatin (CDDP) plus GEM versus GEM for advanced BTC were reported (7) and GEM and CDDP combination therapy showed superiority to GEM monotherapy. Similar results were also reported in Japanese Phase II study (8). CDDP and GEM combination therapy is now considered as a standard first-line regimen for advanced BTC. In 2011, CDDP received approval from social insurance in Japan for advanced BTC.

No standard therapy was established following the progression to GEM-containing first-line therapy. S-1 is an oral agent consisting of a mixture of tegafur, 5-chloro-2, 4-dihydroxypyrimidine and potassium oxonate at a molar ratio of 1:0.4:1 (9), which has mainly been investigated in Asian countries. In a Phase II study of S-1 as a drug for first-line chemotherapy for advanced BTC, it was reported that the objective response rate was 32.5%, and the median survival time (MST) was 9.4 months with median time to progression (TTP) 3.7 months (10,11). Because of the good anti-tumor activity, two prospective studies of S-1 monotherapy as second-line therapy after the progression to GEM (12,13) were conducted. In these studies, the objective response rates were 22.7 and 7.5% and the values of MST were 13.5 and 7.5 months. S-1 is practically used as a drug for second-line chemotherapy in Japan to treat advanced BTC.

However, these results were quite different from one another. Consequently, the efficacy and safety of S-1 monotherapy as second-line therapy for advanced BTC is still not established in a practical setting, which is why we performed this retrospective analysis.

PATIENTS AND METHODS

Patients

The subjects were 55 consecutive patients who received S-1 monotherapy as second-line chemotherapy after the failure to GEM-containing regimen at Kanagawa Cancer Center between September 2007 and March 2011. We retrospectively reviewed their medical records. All the patients received a pathological and graphical diagnosis of BTC (intrahepatic or extrahepatic cholangiocarcinoma, gallbladder cancer or ampullary carcinoma). Advanced BTC was defined as (i) metastasis to other organs or to a distant lymph node, (ii) metastasis to form a bulky lymph node of hepatoduodenal ligament, (iii) invasion to common hepatic artery or proper hepatic artery or celiac artery or superior mesenteric artery, (iv) invasion to the bilateral branches of hepatic artery, (v) invasion to the trunk of portal vein which leads to the growth of collateral vessels, or invasion to the bilateral branches of portal vein, (vi) invasion to the bilateral secondary branch of the bile duct, (vii) invasion to one side of the hepatic artery/portal vein and invasion to another side of the secondary branches of the bile duct and (viii) recurrence after curative surgery. In addition to these criteria, intrahepatic cholangiocarcinoma with intrahepatic metastasis in the bilateral lobe is also defined as advanced BTC. Additional criteria for this retrospective analysis included an Eastern Cooperative Oncology Group performance status (PS) of 0–2, good bone marrow function, white blood cell count $\geq 3000/mm^3$, neutrophil count $\geq 1500/mm^3$, hemoglobin ≥ 8.5 g/dl, platelet count $\geq 100\,000/mm^3$, good renal function (serum creatinine ≤ 1.5 mg/dl) and good liver function (total bilirubin ≤ 2.0 mg/dl and transaminase levels ≤ 2.5 times the upper limit of the normal ranges). Patients with obstructive jaundice were eligible after receiving adequate biliary drainage and decreasing transaminase levels (less than five times the upper limit of the normal range). Patients were excluded if they had not received GEM in the first-line regimen or had already received S-1, or if they had massive ascites, pleural effusion, active concomitant malignancy, brain metastasis, interstitial pneumonia, uncontrolled diabetes mellitus and regular use of warfarin, phenytoin or frucitocin.

Treatment

S-1 was administered orally twice a day at a dose of 40 mg/m^2. The initial doses were determined according to the body surface area (BSA) calculated by body weight and height as follows: BSA $< 1.25\ m^2$, 80 mg/day; $1.25\ m^2 \leq$ BSA $< 1.5\ m^2$, 100 mg/day; $1.5\ m^2 \leq$ BSA, 120 mg/day. S-1 was given for 28 days followed by 14 days of rest. Dose reduction and interruption were considered in the case of severe toxicities (graded as 3–4) according to the Common Terminology Criteria of Adverse Event version 4.0 (CTCAE v4.0). No dose re-escalation was conducted following the dose reduction. This treatment course was repeated until disease progression, unacceptable toxicities or patients' refusal.

Evaluation

Tumor response was assessed approximately every 2 months in contrast-enhanced computed tomography according to the Response Evaluation Criteria In Solid Tumor (RECIST, version 1.1). Toxicities were evaluated according to the CTCAE v4.0. Overall survival was defined as the duration from the date of treatment initiation to the date of death of any cause or the last follow-up. Progression-free survival (PFS) was defined as the duration from the date of S-1 treatment initiation to the date of documented disease progression